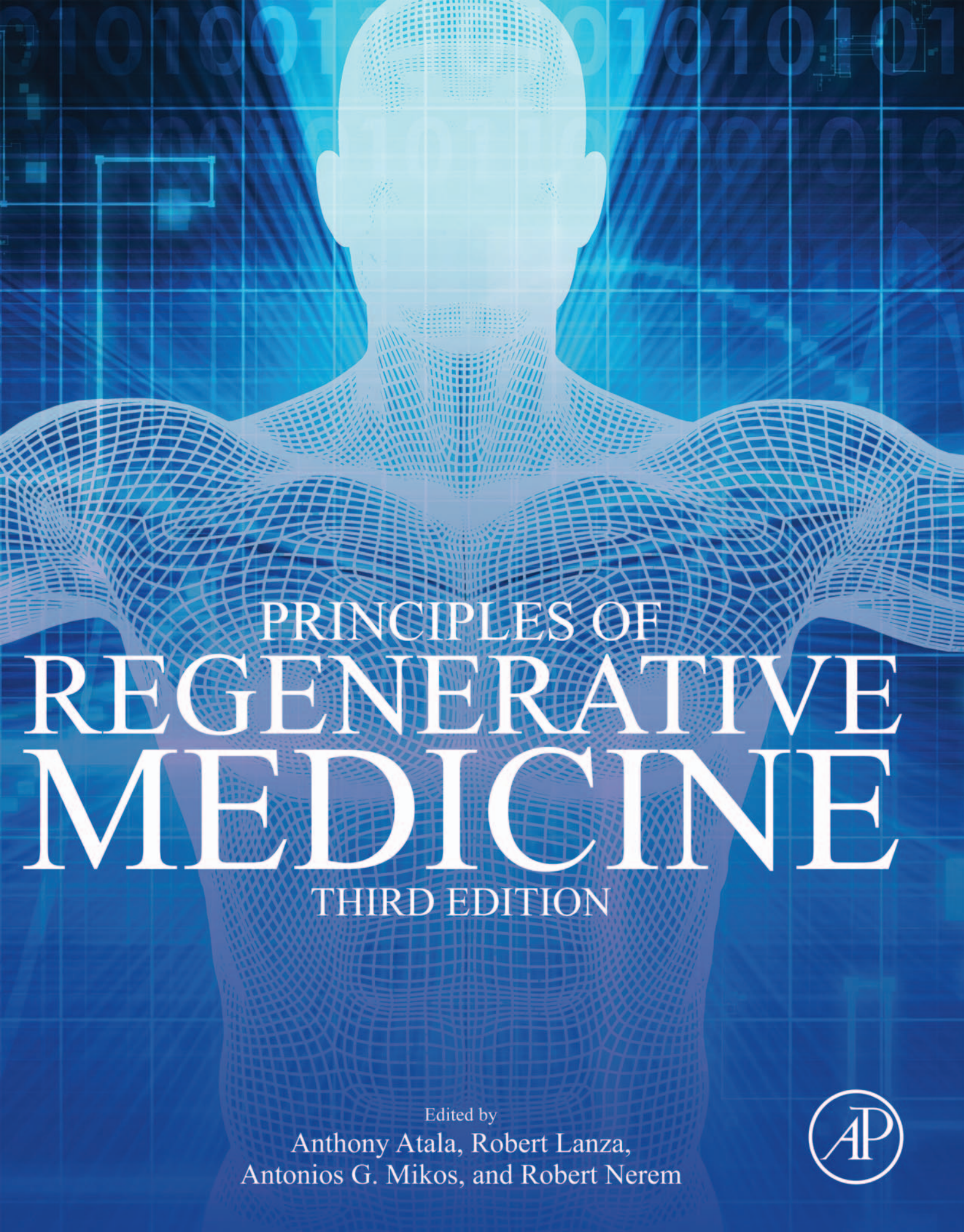
PRINCIPLES OF
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THIRD EDITION
Edited by
Anthony Atala, Robert Lanza,
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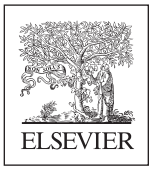

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This book is dedicated to my family: Katherine, Christopher, and Zachary

Anthony Atala

To my family: Mary, Georgios, and Lydia

Antonios G. Mikos

To my wife, Marilyn, and to my four children, Nancy Black, Christy Maser, Steve Nerem, and Carol Wilcox

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Preface

The textbook *Principles of Regenerative Medicine* was created as a primary resource for scientists, clinicians, teachers, and students from academia, industry, and government, as well as the public at large. The initial edition was the first comprehensive body of work dedicated entirely to the field and quickly became the most relevant textbook in the arena of regenerative medicine. The first and second editions of the textbook have had broad appeal and are currently ranked as the most distributed textbooks in the field. I am honored to have had the opportunity to continue to edit the textbook, now in its third edition, with our coeditors, Robert Lanza, Tony Mikos, and Robert Nerem. We welcome Tony Mikos as a new editor and would like to thank Jamie Thomson for his contributions to the first two editions. The contributions of the editors cannot be overestimated. We are indebted to their vision and the strong foundation they have created, upon which the current text was built.

The specialty of regenerative medicine continues to grow and change rapidly. There have been major areas of advances in just the past few years. The field encompasses multiple areas of scientific inquiry, each of which is complex, but together they are a powerful combination of technologies, such as stem cells, gene editing, nuclear transfer, proteomics, pharmacology, nanotechnology, tissue engineering, three-dimensional printing, and biomanufacturing. We are now in an era of translation of bench site discoveries to clinical therapies. We hope that this book will enlighten all of these areas and supply guidance where it is most needed.

The textbook was organized in a manner that builds upon the basic science of the field and goes forward into clinical applications and possible clinical utility. The textbook is organized into seven major areas, starting with chapters that encompass some of the fundamentals of the field. The biologic and molecular bases of regenerative medicine are covered with the molecular, mechanistic, and phenotypic aspects of cells and the extracellular matrix. The second section encompasses cells and tissue development, dealing with the various types of cells and determinants of tissue formation. The third section explores the mechanical, physical, and morphogenetic aspects of regenerative medicine, including nanotechnology applications. The fourth section is dedicated to the area of biomaterials for regenerative medicine. The fifth section covers enabling technologies for regenerative medicine, including infection control, gene editing, preclinical models, bioreactors, bioprinters, and body-on-a-chip applications. The sixth section discusses the topics of therapeutic applications and deals mostly with specific tissue and organ types. The last section of the book is dedicated to the regulatory, manufacturing, policy, and ethical aspects of the field. This area is becoming increasingly more important because the nexus between science, safety, and ethics is constantly changing. The third edition of the *Principles of Regenerative Medicine* builds on the knowledge base of the prior editions; most relevant chapters have been updated and other relevant topics are added with the inclusion of new chapters.

Once again, the leading scientists and physicians in the field have contributed their expertise to this comprehensive collection. We would like to thank all of the authors for their contributions; the publisher, Elsevier, and its staff, including Timothy Bennett, who worked diligently to complete the book; and our commissioning editors, Micah Lewis and Elizabeth Brown, who nurtured this project from its concept to completion.

Anthony Atala
(on behalf of the Editors)

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CHAPTER

1

Molecular Organization of Cells

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INTRODUCTION

Multicellular tissues exist in one of two types of cellular arrangements, epithelial or mesenchymal. Epithelial cells adhere tightly to each other at their lateral surfaces and to an organized extracellular matrix (ECM) at their basal domain, thereby producing a sheet of cells resting on a basal lamina with an apical surface. Mesenchymal cells, in contrast, are individual cells with a bipolar morphology that are held together as a tissue within a three-dimensional (3D) ECM (Fig. 1.1). The conversion of epithelial cells into mesenchymal cells, an "epithelial–mesenchymal transition" (EMT), is central to many aspects of embryonic morphogenesis and adult tissue repair, as well as a number of disease states [1–3]. The reverse process whereby mesenchymal cells coalesce into an epithelium is a "mesenchymal–epithelial transition" (MET). Understanding the molecules that regulate this transition between epithelial and mesenchymal states offers important insights into how cells and tissues are organized.

The early embryo is structured as one or more epithelia. An EMT allows the rearrangements of cells to create additional morphological features. Well-studied examples of EMTs during embryonic development include gastrulation in *Drosophila* [3], the emigration of primary mesenchyme cells (PMCs) in sea urchin embryos [4], and gastrulation in amniotes (reptiles, birds, and mammals) at the primitive streak [2]. EMTs also occur later in vertebrate development, such as during the emigration of neural crest cells from the neural tube [5], the formation of the sclerotome from epithelial somites, and palate fusion [2]. The reverse process of MET is likewise crucial to development; examples include the condensation of mesenchymal cells to form the notochord and somites [6], kidney tubule formation from nephrogenic mesenchyme [7], and the creation of heart valves from cardiac mesenchyme [8]. In the adult organism, EMTs and METs occur during wound healing and tissue remodeling [9]. The conversion of neoplastic epithelial cells into invasive cancer cells has long been considered an EMT process [1,10]. However, there are also examples of tumor cells that have functional cell–cell adhesion junctions yet are still migratory and invasive as a group [11]. This "collective migration" also occurs during development [11]. Hence, there is debate whether an EMT model accurately describes all epithelial metastatic cancers. Similarly, the fibrosis of cardiac, kidney, lens, and liver epithelial tissue has also long been categorized as an EMT event [6,12]. However, research in the kidney in vivo shows that the myofibroblasts induced after kidney injury are derived from mesenchymal pericytes rather than the proximal epithelial cells [13]. Therefore, defining the origin of the cells that contribute to fibrotic tissue scarring (epithelial or otherwise) will require further investigation.

The focus of this chapter is on the molecules that regulate the organization of cells into epithelium or mesenchyme. We will first discuss the cellular changes that occur during an EMT, including changes in cell–cell and cell–ECM adhesions, changes in cell polarity, and the stimulation of invasive cell motility. Then we will consider the molecules and mechanisms that control the EMT or MET, including the structural molecules, transcription factors, and signaling pathways that regulate EMTs.

MOLECULES THAT ORGANIZE CELLS

The conversion of an epithelial sheet into individual migratory cells and back again requires the coordinated changes of many distinct families of molecules.

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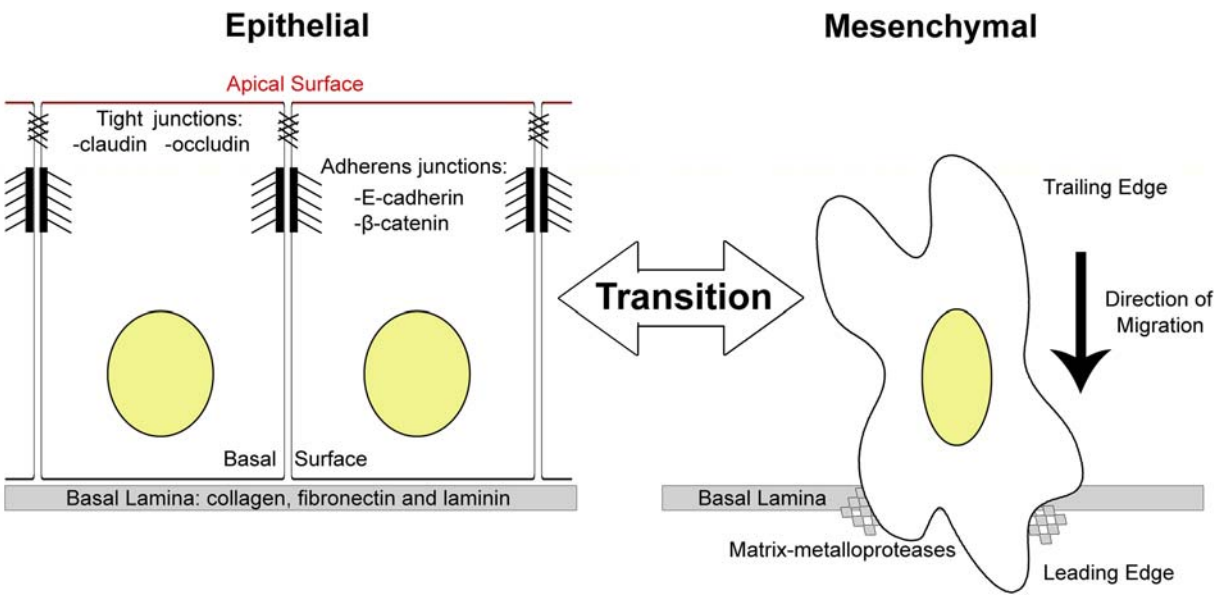

FIGURE 1.1 **Epithelial versus Mesenchymal.** Epithelial cells adhere tightly together by tight junctions and adherens junctions localized near the apical surface. Epithelial cells also have a basal surface that rests on a basal lamina. In contrast, mesenchymal cells do not have well-defined cell–cell adhesion complexes; they have front-end/back-end polarity instead of apicobasal polarity, and mesenchymal cells are characterized by their ability to invade the basal lamina.

Changes in Cell–Cell Adhesion

Epithelial cells are held together by specialized cell–cell junctions, including adherens junctions, desmosomes, and tight junctions [14]. These junctions are localized in the lateral domain near the apical surface and establish the apical polarity of the epithelium. For an epithelial sheet to produce individual mesenchymal cells, cell–cell adhesions must be disrupted. The principal transmembrane proteins that mediate cell–cell adhesions are members of the cadherin superfamily [15]. E-cadherin and N-cadherin are classical cadherins that interact homotypically through their extracellular immunoglobulin G domains with like-cadherins on adjacent cells. Cadherins are important mediators of cell–cell adhesion. For example, misexpression of E-cadherin is sufficient to promote cell–cell adhesion and assembly of adherens junctions in fibroblasts [16]. In epithelial cancers (carcinomas), E-cadherin acts as a tumor suppressor [10]. In a mouse model for β-cell pancreatic cancer, the loss of E-cadherin is the rate-limiting step for transformed epithelial cells to become invasive [17]. Although the loss of cadherin-mediated cell–cell adhesion is necessary for an EMT, the loss of cadherins is not always sufficient to generate a complete EMT in vivo. For example, the neural tube epithelium in mice expresses N-cadherin, but in the N-cadherin knockout mouse an EMT is not induced in the neural tube [18]. Hence, cadherins are essential for maintaining epithelial integrity, and the loss of cell–cell adhesion caused by the reduction of cadherin function is an important step for an EMT.

One characteristic of an EMT is "cadherin switching." Often, epithelia that express E-cadherin will downregulate E-cadherin expression at the time of the EMT and express different cadherins such as N-cadherin [19]. Cadherin switching may promote motility. For instance, in mammary epithelial cell lines, the misexpression of N-cadherin is sufficient for increased cell motility. However, blocking N-cadherin expression does not result in motility even though the adherens junctions are reduced. Hence, cadherin switching may be necessary for cell motility, but cadherin switching alone is not sufficient to bring about a complete EMT [20].

There are several ways in which cadherin expression and function are regulated. Transcription factors that are central to most EMTs, such as Snail-1, Snail-2, Zinc finger E-box-binding (Zeb)1, Zeb2, Twist, and E2A, all bind to E-boxes on the *E-cadherin* promoter and repress the transcription of *E-cadherin* [21]. Posttranscriptionally, the E-cadherin protein is ubiquitinated by the E3-ligase, Hakai, which targets E-cadherin to the proteasome [22]. E-cadherin turnover at the membrane is regulated by either caveolae-dependent endocytosis or clathrin-dependent endocytosis [23], and p120-catenin prevents endocytosis of E-cadherin at the membrane [24]. E-cadherin function can also be disrupted by matrix metalloproteases, which degrade the extracellular domain of E-cadherin [25]. Some or all of these mechanisms may occur during an EMT to disrupt cell–cell adhesion.

Cell–cell adhesion is maintained principally by cadherins, and changes in cadherin expression are typical of an EMT.

Changes in Cell–Extracellular Matrix Adhesion

Altering the way in which a cell interacts with the ECM is also important in EMTs. For example, at the time that sea urchin PMCs ingress, the cells have increased adhesiveness for ECM [4]. Cell–ECM adhesion is mediated principally by integrins. Integrins are transmembrane proteins composed of two noncovalently linked subunits, α and β, that bind to ECM components such as fibronectin, laminin, and collagen. The cytoplasmic domain of integrins links to the cytoskeleton and interacts with signaling molecules. Changes in integrin function are required for many EMTs, including neural crest emigration [26], mouse primitive streak formation [2], and cancer metastasis [27]. However, the misexpression of integrin subunits is not sufficient to bring about a full EMT in vitro [28] or in vivo [29].

The presence and function of integrins are modulated in several ways. For example, the promoter of the *integrin β6* gene is activated by the transcription factor Ets-1 during colon carcinoma metastasis [30]. Most integrins can also cycle between "on" (high-affinity) or "off" (low-affinity) states. This "inside-out" regulation of integrin adhesion occurs at the integrin cytoplasmic tail [31]. In addition to integrin activation, the "clustering" of integrins on the cell surface affects the overall strength of integrin–ECM interactions. The increased adhesiveness of integrins caused by clustering, known as avidity, can be activated by chemokines and depends on RhoA and phosphatidylinositol 3′ kinase (PI3K) activity [31].

Changes in ECM adhesion are required for an EMT. Cell–ECM adhesions are maintained by integrins, which have varying degrees of adhesiveness depending on the presence, activity, or avidity of the integrin subunits.

Changes in Cell Polarity and Stimulation of Cell Motility

Cellular polarity is defined by the distinct arrangement of cytoskeletal elements and organelles in epithelial versus mesenchymal cells. Epithelial polarity is characterized by cell–cell junctions found near the apicolateral domain (nonadhesive surface), and a basal lamina opposite of the apical surface (adhesive surface). Mesenchymal cells, in contrast, do not have apicobasal polarity, but rather front-end/back-end polarity, with actin-rich lamellipodia and Golgi localized at the leading edge [2]. Molecules that establish cell polarity include Cdc42, PAK1, PI3K, PTEN, Rac, Rho, and the PAR proteins [32,33]. Changes in cell polarity help to promote an EMT. In mammary epithelial cells, the activated transforming growth factor-β (TGF-β) receptor II causes Par6 to activate the E3 ubiquitin ligase Smurf1, which then targets RhoA to the proteasome. The loss of RhoA activity results in the loss of cell–cell adhesion and epithelial cell polarity [34].

For mesenchymal cells to leave the epithelium, they must become motile. Many of the same polarity (Crumbs, PAR, and Scribble complexes), structural (actin and microtubules), and regulatory molecules (Cdc42, Rac1, and RhoA) that govern epithelial polarity are also central to cell motility [35]. Cell motility mechanisms also vary depending on whether the environment is two-dimensional or 3D [36]. Many mesenchymal cells express the intermediate filament vimentin, which may be responsible for several aspects of the EMT phenotype [37].

In short, a wide variety of structural, polarity, and regulatory molecules must be reassigned as cells transition between epithelial polarity and mesenchymal migration.

Invasion of the Basal Lamina

In most EMTs, the emerging mesenchymal cells must penetrate a basal lamina, which consists of ECM components such as collagen type IV, fibronectin, and laminin. The basal lamina functions to stabilize the epithelium and is a barrier to migratory cells [38]. One mechanism that mesenchymal cells use to breach the basal lamina is to produce enzymes that degrade it. Plasminogen activator is one protease associated with a number of EMTs, including neural crest emigration [38] and the formation of cardiac cushion cells during heart morphogenesis [39]. The type II serine protease TMPRSS4 also promotes an EMT and metastasis when overexpressed in vitro and in vivo [40]. Matrix-metalloprotease (MMPs) are also important for many EMTs. When MMP-2 activity is blocked in the neural crest EMT, neural crest emigration is inhibited, but not neural crest motility [41]. In mouse mammary cells, MMP-3 overexpression is sufficient to induce an EMT in vitro and in vivo [42]. Misexpressing MMP-3 in cultured cells induces an alternatively spliced form of Rac1 (Rac1b), which then causes an increase in reactive oxygen species (ROS) intracellularly, and Snail-1 expression. Either Rac1b activity or ROS is necessary and sufficient for an MMP-3–induced EMT [43]. Hence, a number of extracellular proteases are important to bring about an EMT.

Although epithelial cells undergoing an EMT eventually lose cell−cell adhesion and apicobasal polarity and gain invasive motility, the EMT program is not necessarily ordered or linear. For example, in a study in which neural crest cells were labeled with cell adhesion or polarity markers and individual live cells were observed to undergo the EMT in slice culture, neural crest cells changed epithelial polarity either before or after the complete loss of cell−cell adhesion, or lost cell−cell adhesion either before or after cell migration commenced [44]. Therefore, whereas an EMT consists of several distinct phases, these steps may occur in different orders or combinations, some of which (e.g., the complete loss of cell-cell adhesion) may not always be necessary.

Changes in a wide range of molecules are needed for an EMT because epithelial cells lose cell−cell adhesion, change cellular polarity, and gain invasive cell motility.

THE EPITHELIAL−MESENCHYMAL TRANSITION TRANSCRIPTIONAL PROGRAM

At the foundation of every EMT or MET program are the transcription factors that regulate the gene expression required for these cellular transitions. Whereas many of the transcription factors that regulate EMTs have been identified, the complex regulatory networks are still incomplete. Here we review the transcription factors that are known to promote the various phases of an EMT. Then we examine how these EMT transcription factors themselves are regulated at the promoter and posttranscriptional levels.

Transcription Factors That Regulate Epithelial−Mesenchymal Transition

The Snail family of zinc-finger transcription factors, including Snail-1 and Snail-2 (formerly Snail and Slug), are direct regulators of cell−cell adhesion and motility during EMTs [21,45]. The knockout of *Snail-1* in mice is lethal early in gestation, and the presumptive primitive streak cells that normally undergo an EMT retain apicobasal polarity and adherens junctions, and express *E-cadherin* messenger RNA [46]. Snail-1 misexpression is sufficient for breast cancer recurrence in a mouse model in vivo, and high levels of *Snail-1* predict the relapse of human breast cancer [47]. Snail-2 is necessary for the chicken primitive streak and neural crest EMTs [48]. One way in which Snail-1 or Snail-2 causes decreases in cell−cell adhesion is by repressing the *E-cadherin* promoter [21]. This repression requires the mSin3A co-repressor complex, histone deacetylases, and components of the Polycomb 2 complex [49]. Snail-1 is also a transcriptional repressor of the tight junction genes *Claudin* and *Occludin* [21] and the polarity gene *Crumbs3* [50]. The misexpression of Snail-1 and Snail-2 further leads to the transcription of proteins important for cell motility, such as fibronectin, vimentin [51], and RhoB [52]. Moreover, Snail-1 promotes invasion across the basal lamina. In Madin−Darby Canine Kidney (MDCK) cells, the misexpression of Snail-1 represses laminin (basement membrane) production [53] and indirectly upregulates *mmp-9* transcription [54]. Snail and Twist also make cancer cells more resistant to senescence, chemotherapy, and apoptosis, and endow cancer cells with "stem cell" properties [6]. Hence, Snail-1 or Snail-2 is necessary and sufficient for bringing about many of the steps of an EMT, including loss of cell−cell adhesion, changes in cell polarity, gain of cell motility, invasion of the basal lamina, and increased proliferation and survival.

Other zinc finger transcription factors important for EMTs are Zeb homeobox 1 (Zeb1; also known as δEF1), and Zeb2 (also known as Smad-interacting protein-1; Sip1). Both Zeb1 and Zeb2 bind to the *E-cadherin* promoter and repress transcription [21]. Zeb1 can also bind to and repress the transcription of the polarity proteins Crumbs3, Pals1-associated tight junction proteins, and Lethal giant larvae 2 [55]. Zeb2 is structurally similar to Zeb1, and Zeb2 overexpression is sufficient to downregulate E-cadherin, dissociate adherens junctions, and increase motility in MDCK cells [56].

The lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) transcription factors also have an important role in EMTs. For instance, the misexpression of Lef-1 in cultured colon cancer cells reversibly causes the loss of cell−cell adhesion [57]. LEF/TCF transcription factors directly activate genes that regulate cell motility, such as the L1 adhesion molecule [58], and the *fibronectin* gene [59]. LEF/TCF transcription factors also upregulate genes required for basal lamina invasion, including *mmp-3* and *mmp-7* [60].

Other transcription factors that have a role in promoting EMTs are the class I basic helix-loop-helix factors E2-2A and E2-2B [61], the forkhead box transcription factor FOXC2 [62], the homeobox protein Goosecoid [63], and the homeoprotein Six1 [64,65].

Transcription factors that regulate an EMT often do so by directly repressing cell adhesion and epithelial polarity molecules and by upregulating genes required for cell motility and basal lamina invasion.

Regulation at the Promoter Level

Given the importance of the Snail, Zeb, and LEF/TCF transcription factors in orchestrating the various phases of an EMT, it is essential to understand the upstream events that regulate these EMT-promoting transcription factors.

The activation of *Snail-1* transcription in *Drosophila* requires the transcription factors Dorsal (nuclear factor κB [NF-κB]) and Twist [21]. The human *Snail-1* promoter also has functional NF-κB sites [66], and blocking NF-κB reduces *Snail-1* transcription [67]. In addition, a region of the *Snail-1* promoter is responsive to integrin-linked kinase (ILK) [21], and ILK can activate Snail-1 expression via poly-adenosine phosphate-ribose polymerase [68]. In mouse mammary epithelial cells, high-mobility group protein A2 and Smads activate *Snail-1* expression and subsequently *Snail-2*, *Twist*, and *Id2* transcription [69]. For *Snail-2* expression, myocardin-related transcription factors (MRTFs) interact with Smads to induce *Snail-2* [70] and MRTFs may have a role in metastasis [71] and fibrosis [72]. There are also several *Snail-1* transcriptional repressors. In breast cancer cell lines, metastasis-associated protein 3 binds directly to and represses the transcription of *Snail-1* in combination with the Mi-2/nucleosome remodeling deacetylase complex [73], as does lysine-specific demethylase [74]. The Ajuba LIM proteins (Ajuba, LIMD1, and WTIP) are additional transcriptional corepressors of the Snail family [75].

The transcription of LEF/TCF genes such as *Lef-1* are activated by Smads [76]. The misexpression of Snail-1 results in the transcription of *δEF-1* and *Lef-1* through a yet unknown mechanism [21].

Posttranscriptional Regulation of Epithelial–Mesenchymal Transition Transcription Factors

The activity of EMT transcription factors is also regulated at the protein level, including translational control, protein stability (targeting to the proteasome), and nuclear localization. Noncoding RNAs are emerging as important regulator EMTs. In a breast cancer model, Myc activates the expression of microRNA-9 (miR-9), and miR-9 directly binds to and represses the *E-cadherin* promoter [77]. Members of the miR-200 family repress the translation of *Zeb1*, and the expression of these miR-200 family members is repressed by Snail-1. In addition, *Zeb2* transcription can be activated by naturally occurring RNA antisense transcripts [78]. It is not yet known whether there are noncoding RNAs that regulate Snail family members. However, the Y-box binding protein-1 is important for the selective activation of *Snail-1* translation [79].

Protein stability is another layer of EMT control. Snail-1 is phosphorylated by glycogen synthase kinase 3β (GSK-3β) and targeted for destruction [80]. Therefore, the inhibition of GSK-3β activity by Wnt signaling may have multiple roles in an EMT, leading to the stabilization of both β-catenin and Snail-1. Some proteins that prevent GSK 3β–mediated phosphorylation (and thus promote Snail-1 activation) are lysyl oxidase–like proteins (LOXL)2, LOXL3 [81], and ILK [82]. A Snail 1–specific phosphatase (Snail-1 activator) is C-terminal domain phosphatase [83]. Snail-2 is targeted for degradation by the direct action of p53 and the ubiquitin ligase Mdm2 [84].

In addition to protein translation and stability, the function of Snail-1 depends on nuclear localization mediated by Snail-1's nuclear localization sequence. The phosphorylation of human Snail-1 by p21-activated kinase 1 promotes the nuclear localization of Snail-1 (and therefore Snail-1 activation) in breast cancer cells [85]. In zebrafish, LIV-1 promotes the translocation of Snail-1 into the nucleus [86]. Snail-1 also contains a nuclear export sequence (NES) that depends on the calreticulin nuclear export pathway [87]. This NES sequence is activated by the phosphorylation of the same lysine residues targeted by GSK-3β, which suggests a mechanism whereby phosphorylation of Snail-1 by GSK-3β results in the export of Snail-1 from the nucleus and subsequent degradation.

LEF/TCF activity is also regulated by other proteins. β-Catenin is required as a cofactor for LEF/TCF-mediated activation of transcription, and Lef-1 can also associate with cofactor Smads to activate the transcription of additional EMT genes [88]. In colon cancer cells, thymosin β4 stabilizes ILK activity [89].

EMT transcription factors such as Snail-1, Zeb1, and Lef-1 are regulated by a variety of mechanisms at both the transcriptional and posttranscriptional levels by noncoding RNA translation control, protein degradation, nuclear localization, and cofactors such as β-catenin.

MOLECULAR CONTROL OF THE EPITHELIAL–MESENCHYMAL TRANSITION

The initiation of an EMT or MET is a tightly regulated event during development and tissue repair because deregulation of cellular organization is disastrous to the organism. A variety of external and internal signaling mechanisms coordinate the complex events of the EMT, and these same signaling pathways are often disrupted or reactivated during disease. EMTs or METs can be induced by either diffusible signaling molecules or ECM

components. We next discuss the role of signaling molecules and ECM in triggering an EMT, and then present a summary model for EMT induction.

Ligand-Receptor Signaling

During development, five main ligand-receptor signaling pathways are employed: TGF-β, Wnt, receptor tyrosine kinase (RTK), Notch, and Hedgehog. These pathways, among others, have a role in triggering EMTs. Although the activation of a single signaling pathway can be sufficient for an EMT, in most cases an EMT or MET is initiated by multiple signaling pathways acting in concert.

Growth Factor-β Pathway

The TGF-β superfamily includes the TGF-β, activin, and bone morphogenetic protein (BMP) families. These ligands operate through receptor serine/threonine kinases to activate a variety of signaling molecules including Smads, mitogen-activated protein kinase (MAPK), PI3K, and ILK. Most EMTs studied to date are induced in part, or solely, by TGF-β superfamily members [90]. During embryonic heart development, TGF-β2 and TGF-β3 have sequential and necessary roles in activating the endocardium to invade the cardiac jelly and from the endocardial cushions [91]. In the avian neural crest, BMP4 induces *Snail-2* expression [92]. In the EMT that transforms epithelial tissue into metastatic cancer cells, TGF-β acts as a tumor suppressor during early stages of tumor development, but as a tumor/EMT inducer at later stages [90,93]. TGF-β signaling may combine with other signaling pathways to induce an EMT. For example, in cultured breast cancer cells, activated Ras and TGF-β induce an irreversible EMT [94], and in pig thyroid epithelial cells, TGF-β and epidermal growth factor (EGF) synergistically stimulate the EMT [95].

One outcome of TGF-β signaling is to change epithelial cell polarity immediately. In a TGF β—induced EMT of mammary epithelial cells, TGF-βRII directly phosphorylates the polarity protein, Par6, leading to the dissolution of tight junctions [34]. TGF-β signaling also regulates gene expression through the phosphorylation and activation of Smads. Smads are important cofactors in the stimulation of an EMT. For example, Smad3 is necessary for a TGF β—induced EMT in lens and kidney tissue in vivo [96]. The Smad3/4 also complex with Snail-1 and co-repress the promoters of cell—cell adhesion molecules [97]. Furthermore, TGF-βRI directly binds to and activates PI3K [98], which in turn activates ILK and downstream pathways.

ILK is emerging as an important positive regulator of EMTs [99]. ILK interacts directly with growth factor receptors (TGF-β, Wnt, or RTK), integrins, the actin skeleton, PI3K, and focal adhesion complexes. ILK directly phosphorylates Akt and GSK-3β, and results in the subsequent activation of transcription factors such as AP-1, NF-κB, and Lef-1. Overexpression of ILK in cultured cells causes the suppression of GSK-3β activity [82], translocation of β-catenin to the nucleus, activation of Lef-1/β-catenin transcription factors, and the downregulation of E-cadherin [100]. Inhibition of ILK in cultured colon cancer cells leads to the stabilization of GSK-3β activity, decreased nuclear β-catenin localization, the suppression of *Lef-1* and *Snail-1* transcription, and reduced invasive behavior of colon cancer cells [101]. ILK activity also results in Lef 1—mediated transcriptional upregulation of MMPs [60]. Hence, ILK (inducible by TGF-β signaling) is capable of orchestrating most of the major events in an EMT, including the loss of cell—cell adhesion and invasion across the basal lamina.

Wnt Pathway

Many EMTs or METs are also regulated by Wnt signaling. Wnts signal through seven-pass transmembrane proteins of the Frizzled family, which activates G-proteins and PI3K, inhibits GSK-3β, and promotes nuclear β-catenin signaling. For example, during zebrafish gastrulation, Wnt11 activates the GTPase Rab5c, which results in the endocytosis of E-cadherin [102]. Wnt6 signaling is sufficient for increased transcription of *Snail-2* in the avian neural crest [103]. Snail-1 expression increases Wnt signaling [104], which suggests a positive feedback loop.

One of the downstream signaling molecules activated by Wnt signaling is β-catenin. β-Catenin is a structural component of adherens junctions. Nuclear β-catenin is also a limiting factor for the activation of LEF/TCF transcription factors. β-Catenin is pivotal for regulating most EMTs. Interfering with nuclear β-catenin signaling blocks the ingression of sea urchin PMCs [105], and in β-catenin mouse knockouts, the primitive streak EMT does not occur and no mesoderm is formed [106]. β-Catenin is also necessary for the EMT that occurs during cardiac cushion development [107]. In breast cancer, β-catenin expression is highly correlated with metastasis and poor survival [108], and blocking β-catenin function in tumor cells inhibits invasion in vitro [109]. It is unclear whether β-catenin overexpression alone is sufficient for all EMTs. If β-catenin is misexpressed in cultured cells, it causes apoptosis [110]. However,

the misexpression of a stabilized from of β-catenin in mouse epithelial cells in vivo results in metastatic skin tumors [111].

Signaling by Receptor Tyrosine Kinase Ligands

The RTK family of receptors and the growth factors that activate them also regulate EMTs or METs. Ligand binding promotes RTK dimerization and activation of the intracellular kinase domains by autophosphorylation of tyrosine residues. These phosphotyrosines act as docking sites for intracellular signaling molecules, which can activate signaling cascades such as Ras/MAPK, PI3K/Akt, JAK/STAT, or ILK. We will cite a few examples of RTK signaling in EMTs and METs.

Hepatocyte growth factor (HGF; also known as scatter factor) acts through the RTK c-met. HGF is important for the MET in the developing kidney [112]. HGF signaling is required for the EMT that produces myoblasts (limb muscle precursors) from somite tissue in the mouse [10]. In epithelial cells, HGF causes an EMT through MAPK and early growth response factor-1 signaling [113].

Fibroblast growth factor (FGF) signaling regulates mouse primitive streak formation [114]. FGF signaling also stimulates cell motility and activates MMPs [115,116].

EGF promotes the endocytosis of E-cadherin [117]. EGF can also increase Snail-1 activity via the inactivation of GSK3-β [118], and EGF promotes increased *Twist* expression through a JAK/STAT3 pathway [119].

Insulin growth factor (IGF) signaling induces an EMT in breast cancer cell lines by activating Akt2 and suppressing Akt1 [120]. In prostate cancer cells, IGF-1 promotes Zeb-1 expression [121]. In fibroblast cells, constitutively activated IGF-IR increases NF-κB activity and Snail-1 levels [122]. In several cultured epithelial cell lines, IGFR1 is associated with the complex of E-cadherin and β-catenin, and the ligand IGF-II causes the redistribution of β-catenin from the membrane to the nucleus, activation of the transcription factor TCF-3, and a subsequent EMT [123].

Another RTK known for its role in EMTs is the ErbB2/HER-2/Neu receptor, whose ligand is heregulin/neuregulin. Overexpression of HER-2 occurs in 25% of human breast cancers, and the misexpression of HER-2 in mouse mammary tissue in vivo is sufficient to cause metastatic breast cancer [124]. Herceptin (antibody against the HER-2 receptor) treatment is effective in reducing the recurrence of HER 2–positive metastatic breast cancers. HER-2 signaling activates *Snail-1* expression in breast cancer through an unknown mechanism [47]. The RTK Axl is also required for breast cancer carcinoma invasiveness [125].

Vascular endothelial growth factor (VEGF) signaling promotes Snail-1 activity by suppressing GSK3-β [126] and results in increased levels of *Snail-1*, *Snail-2*, and *Twist* [127]. Snail-1 can also activate the expression of VEGF [128]. RTK signaling is important for many EMTs.

Notch Pathway

The Notch signaling family also regulates EMTs. When the Notch receptor is activated by its ligand Delta, an intracellular portion of the Notch receptor ligand is cleaved and transported to the nucleus, where it regulates target genes. Notch1 is required for cardiac endothelial cells to undergo an EMT to make cardiac cushions, and the role of Notch may be to make cells competent to respond to TGF-β2 [129]. In the avian neural crest EMT, Notch signaling is required for the induction and/or maintenance of *BMP4* expression [130]. Similarly, Notch signaling is required for the TGF β–induced EMT of epithelial cell lines [131], and Notch promotes *Snail-2* expression in cardiac cushion cells [132] and cultured cells [133].

Hedgehog Pathway

The hedgehog pathway is also involved in EMTs. Metastatic prostate cancer cells express high levels of hedgehog and *Snail-1*. If prostate cancer cell lines are treated with the hedgehog-pathway inhibitor, cyclopamine, levels of *Snail-1* are decreased. If the hedgehog-activated transcription factor, Gli, is misexpressed, *Snail-1* expression increases [134].

Additional Signaling Pathways

Other signaling pathways that activate EMTs include inflammatory signaling molecules, lipid hormones, ROS species, and hypoxia. Interleukin-6 (inflammatory and immune response) can promote Snail-1 expression in breast cancer cells [135], and Snail-1, in turn, can activate interleukin-6 expression [136], providing a link between inflammation and EMTs [137]. The lipid hormone prostaglandin E2 (PGE2) induces Zeb1 and Snail activity in lung cancer cells [138], and Snail-1 can also induce PGE2 expression [139]. ROS species can also activate EMTs by PKC and

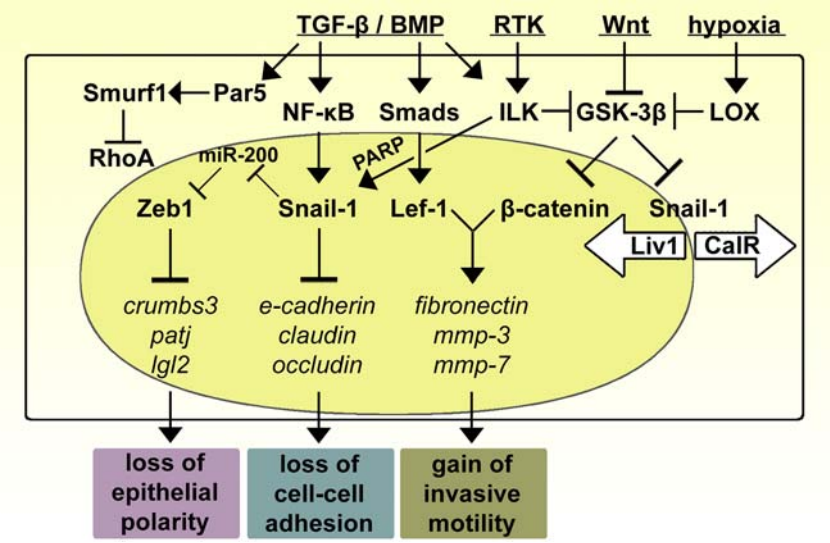

FIGURE 1.2 **Induction of an epithelial–mesenchymal transition (EMT).** This figure summarizes some of the important molecular pathways that bring about an EMT. Many of the signaling pathways converge on the activation of Snail-1 and nuclear β-catenin signaling to change gene expression, which results in the loss of epithelial cell polarity, the loss of cell–cell adhesion, and increased invasive cell motility. *BMP*, bone morphogenetic protein; *CalR*, calreticulin; *GSK-3β*, glycogen synthase kinase 3β; *Igl2*, immunoglobulin 2; *ILK*, integrin-linked kinase; *LOX*, lysyl oxidase; *miR-200*, microRNA-200; *mmp*, matrix-metalloprotease; *NF-κB*, nuclear factor κB; *RhoA*, Ras homolog gene family, member A; *RTK*, receptor tyrosine kinase; *TGF-β*, transforming growth factor-β; *Zeb1*, zinc finger E-box-binding homeobox 1.

MAPK signaling [140]. Hypoxia is important for initiating EMTs during development [141] and disease [137], often through hypoxia-inducible factor-1, which directly activates *Twist* expression [142]. Hypoxia also activates lysyl oxidases, which stabilize Snail-1 expression [143] by inhibiting GSK-3β activity [144].

In addition to diffusible signaling molecules, ECM molecules regulate EMTs or METs. This was first dramatically demonstrated when lens or thyroid epithelium was embedded in collagen gels, and then promptly underwent an EMT [2]. Integrin signaling appears to be important in this process [145] and involves ILK-mediated activation of NF-κB, Snail-1, and Lef-1 [146]. Other ECM components that regulate EMTs include hyaluronan [147], the γ-2 chain of laminin 5 [148], periostin [149], and podoplanin [150,151]. A variety of diffusible signals and ECM components can stimulate EMTs or METs.

A Model for Epithelial–Mesenchymal Transition Induction

Many experimental studies on EMT mechanisms are piecework, and although great progress has been made in discovering EMT pathways, the entire signaling network is still incomplete. Fig. 1.2 shows many of the various signaling mechanisms, although in actuality only a few of the inductive pathways will be used for individual EMTs. From experimental evidence, it appears that many EMT signaling pathways converge on ILK, the inhibition of GSK-3β, and stimulation of nuclear β-catenin signaling to activate Snail and LEF/TCF transcription factors. Snail, Zeb, and LEF/TCF transcription factors then act on a variety of targets to suppress cell–cell adhesion, induce changes in cell polarity, stimulate cell motility, and promote invasion of the basal lamina.

CONCLUSION

Since the term "EMT" was coined [10], important insights have been made in this rapidly expanding field of research. EMT and MET events occur during development, tissue repair, and disease, and many molecules that regulate the various EMTs or METs have been characterized, thanks in large part to the advent of cell culture models. However, the EMT regulatory network as a whole is still incomplete. An improved understanding of EMT and MET pathways will lead to more effective strategies for tissue engineering and novel therapeutic targets for the treatment of disease.

List of Acronyms and Abbreviations

BMP Bone morphogenetic protein
ECM Extracellular matrix
EGF Epidermal growth factor
EMT Epithelial−mesenchymal transition
FGF Fibroblast growth factor
GSK-3β Glycogen synthase kinase 3β
HGF Hepatocyte growth factor
IGF Insulin growth factor
ILK Integrin-linked kinase
LEF/TCF Lymphoid enhancer-binding factor/T-cell factor
LOXL proteins Lysyl oxidase−like proteins
MDCK cells Madin−Darby Canine Kidney cells
MET Mesenchymal-epithelial transition
MMPs Matrix-metalloproteases
MRTFs Myocardin-related transcription factors
NES Nuclear export sequence
PGE2 Prostaglandin E2
PI3K Phosphatidylinositol 3 kinase
PMC Primary mesenchyme cells
ROS Reactive oxygen species
RTK Receptor tyrosine kinase
TGF-β Transforming growth factor-β
VEGF Vascular endothelial growth factor
Zeb Zinc finger E-box-binding

Glossary

Apical Surface of the epithelial layer where adherens junctions and tight junctions are located. This is opposite the basal surface.
Basal Surface of the epithelial layer where the basal lamina is found. This is opposite the apical surface.
Basal Lamina Consists of extracellular matrix components such as collagen type IV, fibronectin, and laminin. The basal lamina functions to stabilize the epithelium and is a barrier against migratory cells.
Epithelial Epithelial cells adhere tightly to each other at their lateral surfaces and to an organized extracellular matrix at their basal domain, thereby producing a sheet of cells resting on a basal lamina with an apical surface.
Epithelial−Mesenchymal Transition The conversion of epithelial cells into mesenchymal cells.
Mesenchymal Mesenchymal cells are individual cells with a bipolar morphology that are held together as a tissue within a three-dimensional extracellular matrix.
Mesenchymal−Epithelial Transition The conversion of mesenchymal cells into epithelial cells.

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CHAPTER

2

Cell–Extracellular Matrix Interactions in Repair and Regeneration

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INTRODUCTION

For many years, the extracellular matrix (ECM) was thought to serve only as a structural support for tissues. Several studies conducted in the latter half of the 20th century dispelled this notion, providing evidence that matrix molecules promote the conversion of myoblasts to myotubes and facilitate the morphogenesis of multiple glands and organs [1]. Data from these and other studies collectively implicated the ECM in embryonic inductions and suggested the presence of matrix binding sites on the surface of cells responding to specific matrix molecules. These ideas opened an entire field of inquiry investigating the detailed mechanisms through which ECM molecules influence cell behavior. Bissell et al. proposed the model of "dynamic reciprocity," in which ECM molecules transmit signals across the cell membrane via cell surface receptors, stimulating signaling pathways that change expression of specific genes whose protein products then affect or alter the ECM [1]. It has become clear that this concept is correct, because cell–ECM interactions activate intracellular signaling, modulate cytokine and growth factor activities, and regulate cell adhesion, migration, growth, differentiation, and programmed cell death.

Much of our current understanding of the molecular basis of cell–ECM interactions in these events comes from in vitro cell or organ cultures and in vivo experiments involving changes in the composition or function of matrix molecules. Here, we will first briefly discuss the composition and diversity of some of the better-known ECM molecules and their receptors, and then discuss selected examples that illustrate the dynamics of cell–ECM interactions during regenerative (scarless) and nonregenerative (scar-forming) wound healing, as well as the potential mechanisms through which matrix-induced signaling affects wound repair. Finally, we will discuss the implications of cell–ECM interactions in regenerative medicine.

COMPOSITION AND DIVERSITY OF THE EXTRACELLULAR MATRIX

The ECM is a molecular complex with many components, including, but not limited to, collagens, hyaluronan, proteoglycans, glycosaminoglycans (GAGs), and elastins. These molecules interact with each other and with growth factors, cytokines, and matrix-degrading enzymes and their inhibitors. The distribution and organization of matrix molecules are not static, but rather vary from tissue to tissue and, during development and tissue repair after injury, from stage to stage, conferring distinct properties and functions on the tissue in question [1,2]. For example, mesenchymal cells are immersed in an interstitial matrix that confers specific biomechanical and functional properties to connective tissue, whereas epithelial and endothelial cells contact the specialized matrix of the basement membrane via only their basal surfaces, conferring mechanical strength and specific physiological properties to the epithelia [3]. The temporal and spatial presence and distribution of specific matrix molecules are critical for the function of both mature tissues and new tissues generated by development or repair, as shown by the impact of mutations in matrix molecules and their associated receptors on these processes [3]. This diversity of composition, organization, and distribution of ECM among different tissues and the same tissue under different conditions results from changes

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in gene expression, splicing, and posttranslational modifications of matrix molecules. For example, alternative splicing or proteolytic cleavage may change the binding potential of matrix proteins to their receptors or to other matrix molecules, whereas altered patterns of glycosylation affect cell adhesion and migration [4–6].

Changes in the structure, organization, and components of the ECM also can affect the distribution and function of growth factors and cytokines that interact with the ECM, influencing downstream signaling in multiple ways. Through its binding to growth factors and cytokines, matrix molecules can protect these molecules from degradation, regulate their local concentrations, facilitate their formation of stable gradients within a tissue, and/or present them more effectively to their receptors, all of which increase downstream signaling and thereby alter signaling-induced cell behaviors [4,7–10]. One example of this matrix-facilitated growth factor signaling involves the binding of vascular endothelial growth factor (VEGF) to fibronectin or heparin sulfate proteoglycans, which increases endothelial proliferation and migration [9]. However, matrix molecules can also bind and sequester growth factors, thereby preventing ligand–receptor interactions and downstream signaling. Binding of heparan sulfate proteoglycans (HSPGs) to heparin-binding endothelial growth factor (EGF)-like factor growth factor (HB-EGF) prevents receptor binding and downstream signaling until proteolytic release of the ligand from the HSPGs [11].

Further complicating the impact of matrix molecules on growth factor signaling, some matrix molecules and matricryptins, proteolytic fragments of matrix molecules, can bind directly to growth factor receptors and either activate or inhibit downstream signaling [4]. For example, specific domains of matrix molecules, including the epidermal growth factor (EGF)-like repeats of laminin-332 and tenascin C, bind and activate the epidermal growth factor receptor (EGFR) [12]. These EGF-like repeats may function as matricryptins after their proteolytic release from their molecules of origin, allowing EGF-like matrix-derived peptides to function as soluble ligands that activate the EGFR, or the EGF-like repeats may function as EGFR ligands in the context of the intact matrix molecule [8,12]. If these EGF-like repeats can induce signaling as part of intact matrix molecules, these repeats could function as persistent, stable inducers of EGFR signaling that could regulate cell function over long periods [8]. In contrast, some matrix molecules and many matricryptins inhibit growth factor receptors, suppressing or antagonizing their downstream effects on cell survival, proliferation, and migration. For example, the proteoglycan decorin binds multiple growth factor receptors and interferes with downstream signaling, whereas fragments of collagen XVIII (endostatin), collagen IV (tumstatin), and the proteoglycan perlecan (endorepellin) bind and inhibit or downregulate VEGF receptors, decreasing endothelial cell survival and migration [10,12].

RECEPTORS FOR EXTRACELLULAR MATRIX MOLECULES

To establish the direct effects of ECM molecules on cell behavior, it was important to identify transmembrane receptors for the specific sequences present on these molecules. Early investigations of salivary gland morphogenesis showed that intracellular microfilaments contracted near the sites of glycosaminoglycan deposition, which suggested that the matrix could regulate microfilament function through the binding of matrix molecules with cell surface receptors [12a]. Later experiments demonstrated that ECM molecules contain specific amino acid motifs enabling their direct binding to cell surface receptors, the best-characterized of which is the tripeptide RGD, first found in fibronectin but later identified in many other matrix molecules [8].

The RGD motifs found in multiple matrix molecules serve as ligands for a subset of receptor proteins called integrins. Integrins, a family of heterodimeric transmembrane proteins composed of α and β subunits, were the first ECM receptors to be identified [13]. The 18 α and 8 β integrin subunits interact with each other in multiple combinations to generate a diverse family of matrix-binding receptors (Fig. 2.1). Some integrins have restricted ligand specificity; others bind multiple epitopes located on the same or different ECM molecules (Fig. 2.1) [13]. In contrast to the extracellular ligand-binding domains of the integrin heterodimers, the intracellular domains of these receptors are relatively small. Despite the relatively short length of their cytoplasmic domains, integrins interact with an array of intracellular signaling proteins that facilitate integrin-associated, matrix-induced signal transduction [14]. The simultaneous binding of some integrins, including integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, to both a matrix ligand and a growth factor receptor attached to its growth factor ligand facilitates growth factor receptor signaling, expanding the role of integrin–matrix binding in the regulation of cell behavior [15].

Although not as extensively studied as the integrins, several proteoglycans, including members of the syndecan family, CD44, and RHAMM (receptor for hyaluronate-mediated motility) also function as ECM receptors [6]. The extracellular domains of syndecans interact with multiple ligands, including growth factors and matrix molecules

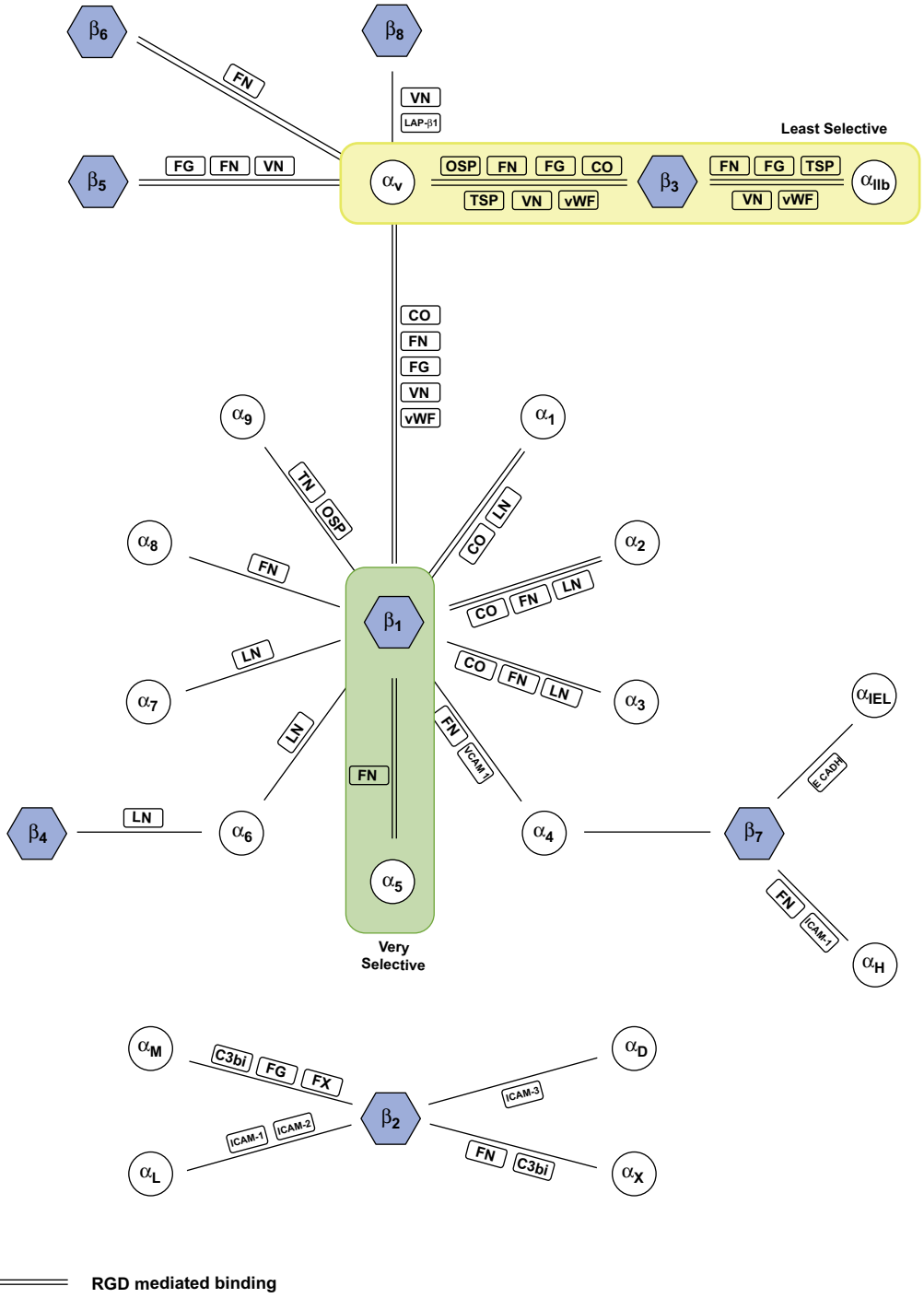

FIGURE 2.1 **Representative members of the integrin family of extracellular matrix (ECM) receptors and their respective ligands.** These heterodimeric receptors are composed of one α and one β subunit and are capable of binding a variety of ligands, including immunoglobulin superfamily cell adhesion molecules, complement factors, and clotting factors, in addition to ECM molecules. Cell–cell adhesion is largely mediated through integrin heterodimers containing the β_2 subunits, whereas cell–matrix adhesion is mediated primarily via integrin heterodimers containing the β_1 and β_3 subunits. In general, the β_1 integrins interact with ligands found in the connective tissue matrix, including laminin, fibronectin, and collagen, whereas the β_3 integrins interact with vascular ligands, including thrombospondin, vitronectin, fibrinogen, and von Willebrand factor. *CO*, collagens; *C3bi*, complement component; *FG*, fibrinogen; *FN*, fibronectin; *FX*, Factor X; *ICAM-1*, intercellular adhesion molecule-1; *ICAM-2*, intercellular adhesion molecule-2; *ICAM-3*, intercellular adhesion molecule-3; *LN*, laminin; *OSP*, osteopontin; *TN*, tenascin; *TSP*, thrombospondin; *VCAM-1*, vascular cell adhesion molecule-1; *VN*, vitronectin; *vWF*, von Willebrand factor.

such as fibronectin and multiple collagens, via chondroitin- and heparan-sulfate GAGs, whose composition varies based on the specific syndecan family member and the tissue in which it is expressed [16]. In contrast to the variable extracellular domain, the syndecan transmembrane and intracellular domains are small and relatively conserved, interacting with the actin cytoskeleton and several associated signaling molecules, including kinases in the Src and PKC families as well as Tiam1, a guanine nucleotide exchange factor for and activator of the Rho GTPases [17]. Although these intracellular signaling molecules are likely responsible for syndecan-mediated cytoskeletal reorganization, syndecan binding to nonmatrix molecules, including growth factors, growth factor receptors, and integrins, makes the identification of matrix-specific, syndecan-mediated signal transduction challenging [16].

Like syndecans, the CD44 receptor carries chondroitin sulfate and heparan sulfate chains on its extracellular domain and undergoes tissue-specific splicing and glycosylation to yield multiple isoforms [6]. Although hyaluronan is its primary ligand, CD44 interacts with other matrix molecules, including fibronectin, laminin, collagen IV, and collagen XIV. Furthermore, the ability of the heparan- and chondroitin-sulfate GAGs on CD44 to bind growth factors, combined with the interactions of CD44 with growth factor receptors, such as EGFR and transforming growth factor-βR (TGFβR), suggests a role for CD44 in modulating growth factor signaling [6,18]. In contrast to the transmembrane CD44 and syndecan proteoglycans, RHAMM, another proteoglycan able to both bind matrix molecules and induce signaling, is associated with the cell membrane through a glycosylphosphatidylinositol (GPI) linkage and not a transmembrane domain. As such, RHAMM located on the cell surface likely activates intracellular signaling through indirect mechanisms, via interactions with transmembrane receptors such as CD44 or growth factor receptors [19]. Interestingly, a variant of RHAMM lacking the GPI anchor resides in the cytoplasm and/or nucleus, where it affects intracellular signaling and cytoskeletal organization through its binding to intracellular signaling molecules and the cytoskeleton, expanding the role of RHAMM as a regulator of cell signaling [19].

Cell surface receptors other than integrins or proteoglycans, including the elastin receptor complex (ERC), CD36, annexin II, Toll-like receptors, and discoidin domain receptors (DDRs) can also serve as receptors for ECM molecules. The ERC is a complex of proteins, including elastin-binding protein (EBP), a splice variant of β-galactosidase, as well as neuraminidase 1 and cathepsin A, that serves as a receptor for elastin, laminin, fibrillin, and peptides derived from these ECM molecules. Signaling activated by this receptor is necessary for elastin deposition and participates in signaling induced by elastin and laminin during mechanotransduction [20]. Another nonintegrin, nonproteoglycan receptor, CD36, better known for its function as a scavenger receptor for long-chain fatty acids and oxidized low-density lipoprotein, binds thrombospondin, collagen I, and collagen IV [21]. CD36-thrombospondin binding activates a variety of signal transduction molecules, ultimately leading to inhibition of angiogenesis via increased endothelial cell apoptosis [22]. Cell surface annexin II, yet another matrix receptor, interacts with alternative splice variants of tenascin-C and facilitates proliferative and migratory effects of these tenascin C splice variants [23]. Although not a matrix receptor per se, Toll-like receptors can function as receptors for fragments of multiple matrix molecules, including fibronectin and low–molecular weight fragments of hyaluronan, which suggests that such fragments may function as danger signals that induce inflammation in response to matrix degradation [18,24].

Receptor tyrosine kinases can also serve as receptors for matrix molecules, as described earlier for EGFR, which binds matrix molecules through their EGF-like domains, as well as the collagen-binding discoidin domain receptors DDR1 and DDR2. Whereas the DDR proteins, like all receptor tyrosine kinases, promote receptor phosphorylation in response to ligand binding, DDRs form constitutive dimers required for ligand binding, unlike most of this receptor family, which dimerize after ligand binding [25]. Also, in contrast to other receptor tyrosine kinase activation, DDR activation by their collagen ligands induces prolonged rather than transient signaling events [26]. The combination of long-lived collagen ligands and the relatively long-term signaling induced by these ligands through the DDRs could provide sustained regulation of cell survival, proliferation, and/or migration in cells expressing these receptors.

In the next section, we will focus on ways in which ECM molecules and their various receptors affect signal transduction, the multiple cell behaviors regulated by this signaling, and the impact of this signaling on wound healing under both nonregenerative and regenerative conditions.

SIGNAL TRANSDUCTION EVENTS DURING CELL–EXTRACELLULAR MATRIX INTERACTIONS

As a result of interactions between ECM molecules and their receptors, as described previously, signals can be transmitted directly or indirectly to signaling molecules within the cell, activating a cascade of events and the coordinated expression of a variety of genes involved in cell adhesion, migration, proliferation, differentiation, and

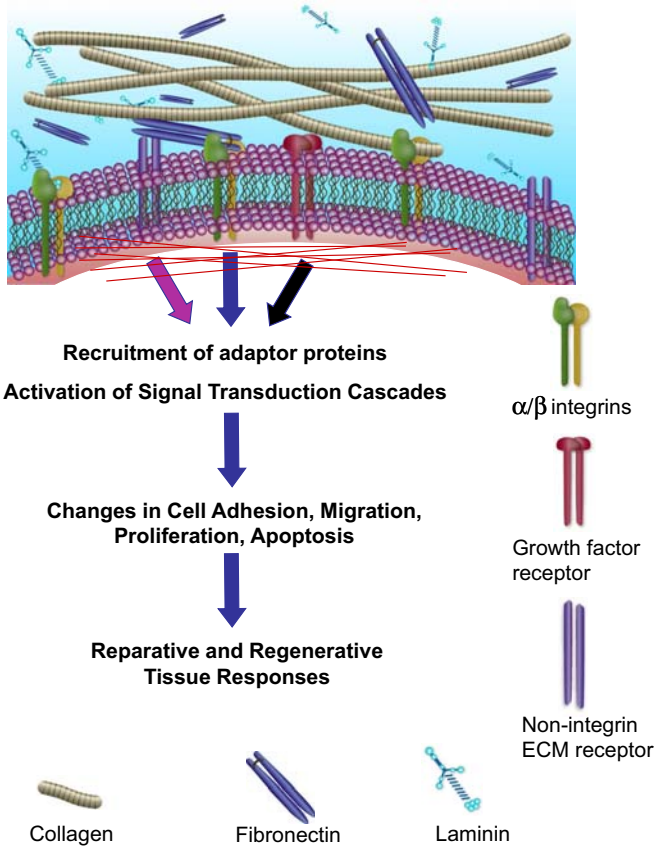

FIGURE 2.2 **Schematic diagram of cell–extracellular matrix (ECM) interactions present during healing and regenerative responses.** Such interactions between the ECM receptors and their respective ligands initiate signal transduction cascades culminating in a variety of cellular events important in repair and regeneration, including changes in cellular adhesion and migration and altered rates of proliferation and apoptosis. The presence and/or extent of such changes may influence the balance of repair and regenerative responses to favor one outcome over another; thus, interventions that alter ECM signaling events may shift this balance to favor tissue regeneration and decrease scarring.

programmed cell death (Fig. 2.2). Some of the increasing evidence that cell–ECM interactions through multiple types of matrix receptors activate a variety of signaling pathways affecting these specific cell outcomes is described subsequently.

Adhesion and Migration

When considering the importance of cell–matrix interactions in adhesion and migration, it is important to recognize that some receptors for ECM molecules, such as the integrins, can participate in both traditional "outside-in" signaling, leading to the activation of intracellular signaling, and "inside-out" signaling, in which intracellular signaling activates the receptor by increasing its affinity for an ECM molecule. This is further complicated by the fact that receptor activation and ligand binding can also initiate outside-in signaling. In the following section, the signaling events refer to outside-in signaling unless otherwise specified.

The outside-in signaling activated by integrin binding to its ligand requires the indirect interaction of the integrin cytoplasmic domain with the cytoskeleton, through a variable group of proteins that collectively comprise the integrin adhesion complex [14]. Some of the proteins of the integrin adhesion complex facilitate integrin-mediated mechanotransduction, whereas other proteins connect the integrins to other types of downstream signal transduction pathways [14]. Integrin-mediated ECM signaling uses these associated proteins to induce changes in cell shape and lead to proliferation, migration, and/or differentiation [27]. Some of the best-known components of the integrin adhesion complex include the signaling adaptor proteins focal adhesion kinase (FAK) and paxillin, the mechanotransducing proteins vinculin and talin, the actin regulatory proteins zyxin and VASP, and the actin-binding protein α-actinin [14]. When the integrin heterodimer interacts with its matrix ligand, FAK, which is associated with the

membrane-proximal portion of the integrin cytoplasmic domain, becomes autophosphorylated on tyrosine 397 (Y387) [28]. The Y397 autophosphorylation may be associated with mechanosensation, as shown by the positive correlation between Y397 phosphorylation and adhesion to fibronectin of increasing stiffness [29]. However, the role of mechanosensation in FAK Y397 phosphorylation may depend on both the matrix molecule and the integrin in question, because soluble collagen but not soluble fibronectin can induce FAK autophosphorylation in suspended cells [29]. Upon integrin–matrix binding and FAK activation, the phosphorylated Y397 serves as a binding site for the SH2 domain of the nonreceptor tyrosine kinase c-Src, which then enhances FAK activity by phosphorylating additional tyrosine residues [28,30]. Src-mediated FAK phosphorylation on tyrosine 925 generates a binding site for the Grb2/Sos complex, with subsequent activation of Ras and the mitogen-activated protein kinase (MAPK) cascade, which may be involved in adhesion/deadhesion and migration [28,31].

In addition to activation of the Ras/MAPK pathway, the FAK/Src complex phosphorylates and regulates the activity of other components of the integrin adhesion complexes, including paxillin, p130Cas, vinculin, and tensin [32]. All of these proteins are associated with cell migration, as shown by the migration defects observed in cells either lacking these molecules altogether and/or expressing mutants affecting their activity or localization [33–36]. For example, paxillin-deficient fibroblasts exhibit both reduced phosphorylation of signaling molecules downstream of integrin ligation and decreased cell motility [33]. The phosphorylation of paxillin by the FAK/Src complex promotes paxillin binding to the SH2 domain of the Crk/DOCK180/ELMO complex. DOCK180, a guanine nucleotide exchange factor (GEF) for Rac1, then activates Rac1 and promotes cell migration [37]. Similarly, p130Cas phosphorylation by the FAK/Src complex promotes Rac1 activation and migration through Crk/DOCK180/ELMO-mediated Rac activation [28]. Beyond their activation of Rac, phosphorylated integrin adhesion complex components regulate other members of the Rho GTPase family. For example, phospho-paxillin also appears to activate p190RhoGAP, leading to localized inhibition of RhoA activity [37]. Additional regulators of RhoA and Rac activity in migrating cells include members of the tensin family, which, like paxillin and p130Cas, are phosphorylated by FAK/Src complexes. In contrast to the RhoGAP-activating function of phosphorylated paxillin, however, tensin1 increases RhoA activity by inhibiting the RhoGAP activity of DLC1 [34]. The conventional understanding of Rac and RhoA dynamics during cell migration suggests that the combination of enhanced Rac1 activity and decreased RhoA activity at the leading edge of migrating cells decreases cell adhesion and promotes protrusion formation, thereby facilitating cell migration. However, Förster resonance energy transfer–based sensors demonstrate both RhoA and Rac activity at the leading edge of migrating cells, suggesting a more complex relationship between these GTPases and membrane protrusion than previously thought and demonstrating the need for more research in this area [38].

Although FAK and c-Src are best known for their roles in outside-in signaling, as described earlier, these kinases are also involved in inside-out signaling, which promotes integrin–ligand binding. FAK promotes integrin activation, cell adhesion to fibronectin, and strengthening of focal adhesions [39]. These effects appear to require Src binding and/or activity, because a Y397 F mutation that prevents FAK autophosphorylation and Src binding at this site also prevents FAK-mediated adhesion [39]. FAK-induced integrin binding to ECM molecules can then initiate outside-in signaling, leading to more FAK activation, FAK-Src interaction, and downstream signaling that promotes deadhesion and migration. This suggests a cycle of FAK and Src activity, in which they initially promote deadhesion and migration, followed by the formation of new adhesions at the leading edge. In support of this FAK/Src cycle of activity, active Src moves from the focal adhesions to the membrane ruffles at the leading edge during cell migration [39a]. The activation of integrins downstream of FAK/Src signaling may mediated by talins and kindlins, two families of proteins that bind directly to the cytoplasmic domains of β integrins. FAK is necessary for talin recruitment to integrins at newly forming adhesion sites, and cells either lacking specific talin family members or expressing mutants that abolish talin–integrin interactions prevent integrin activation induced by various stimuli, leading to defects in integrin-mediated adhesion [40]. Deficiencies in kindlin expression are associated with similar defects in integrin activation and cell adhesion, which suggests the possibility that talins and kindlins work together, potentially downstream of FAK, to activate integrins and promote cell adhesion [41]. However, kindlin signaling is not limited to inside-out signaling; it also participates in outside-in signaling after integrin engagement with matrix ligands. The interaction of kindlin-2 with Src is not required for inside-out signaling and cell adhesion to fibronectin but is necessary for paxillin phosphorylation downstream of integrin ligation and for platelet-derived growth factor (PDGF)-induced mesangial cell proliferation and migration [42]. These findings suggest that kindlin-2 may have multiple roles in integrin-mediated signaling, both promoting integrin-mediated adhesion and facilitating integrin-induced signaling after matrix adhesion, underscoring the interplay between inside-out and outside-in integrin signaling.

A separate form of integrin activation occurs downstream of growth factor receptors. VEGF binding to the VEGFR2 in endothelial cells activates c-Src, which directly phosphorylates β_3 integrin, increasing endothelial cell

adhesion to the $\alpha_v\beta_3$ ligand vitronectin [43]. Interestingly, Src is also necessary for integrin $\alpha_v\beta_3$ interaction with VEGFR2, which is also required for maximal VEGFR2 phosphorylation and VEGF-induced endothelial cell migration in vitro and angiogenesis in vivo [43]. Furthermore, VEGF-induced endothelial cell migration requires FAK activity [44]. These data suggest that the interactions between VEGFR2 and integrin $\alpha_v\beta_3$ couple integrin activation with VEGFR2 signaling, promoting FAK activation and signaling downstream of FAK (described earlier), thereby linking cell–matrix adhesion with promigratory signaling.

Beyond integrin signaling in cell adhesion and migration, nonintegrin ECM receptors, including proteoglycan receptors such as syndecans, CD44, and RHAMM, as well as the elastin-laminin receptor, the EGFR, and DDR1, also participate in cell adhesion and migration through a variety of mechanisms. For example, syndecans facilitate migration through both activation of signaling through the cytoplasmic domains of the syndecans themselves and syndecan-mediated activation of growth factor receptors and integrins [17]. The PDZ-binding motif, located on syndecan intracellular domains, binds several signaling proteins with PDZ domains, including the Rac1 GEF Tiam1, which suggests a mechanism by which syndecan binding to matrix ligands directly regulates cell migration [17]. Syndecans also collaborate with integrins and growth factor receptors in the induction of cell adhesion and migration. In the case of integrins, syndecan-4 regulates the formation and generation of specific integrin adhesion complexes by promoting the internalization and degradation of some integrin heterodimers while increasing surface levels of different heterodimers [16,45]. These alterations in integrin composition induced by syndecan-4 may affect both cell adhesion and integrin-induced signaling important in cell migration. In support of this idea, syndecan cooperation with integrin heterodimers mediates cell adhesion to vitronectin and laminin and induces cell migration, and syndecan-4 promotes Rac1 activation and localization to the leading edge of migrating cells, facilitating directional cell migration in response to fibronectin [46]. Syndecans also participate in growth factor receptor signaling through a complex web of interactions in which the syndecans bind growth factors and prevent receptor binding until proteolytic release (e.g., HB-EGF) or bind both growth factor and its receptor to promote receptor activation (e.g., fibroblast growth factor [FGF] and VEGF) [16]. These growth factor–proteoglycan interactions may provide a stable growth factor gradient that facilitates directional cell migration [8,9].

Two non-syndecan proteoglycan receptors, CD44 and RHAMM, both bind hyaluronan and collaborate to promote hyaluronan-induced signaling. Hyaluronan is a matrix molecule that is generally produced in a high–molecular weight form that can cross-link its cell surface receptors, promoting cell adhesion and inhibiting cell migration [19]. However, lower–molecular weight hyaluronan fragments found in injured and/or inflamed tissues promote cell migration associated with inflammation and angiogenesis [47]. Some of these differences in function between high– and low–molecular weight hyaluronan likely result from differences in receptor selection, because low–molecular weight forms are more likely to bind Toll-like receptors, which can then promote inflammation through nuclear factor κB–induced expression of cytokines and chemokines [47]. However, the angiogenesis induced by hyaluronan fragments requires CD44, which suggests that hyaluronan fragments of different sizes may induce different conformational changes in CD44 or selectively bind different CD44 splice or glycosylation variants, in either case inducing different downstream signaling pathways that either promote angiostatic or angiogenic outcomes [47,48]. Some of these CD44 variants can interact with growth factors and/or their receptors, modulating downstream signaling. For example, the splice variant CD44v6 binds both to VEGF isoforms and to VEGFR2, and CD44v6 inhibition decreases VEGF-induced activation of VEGFR2 and downstream phosphorylation of ERK in endothelial cells [49]. Furthermore, CD44v6 inhibition significantly decreases VEGF-induced endothelial cell migration in vitro and angiogenesis in vivo, which underscores the importance of this CD44 variant in promigratory VEGFR2 signaling [49].

Fragments of matrix components, likely generated under proteolytic conditions such as those occurring during wound healing and inflammation, bind multiple matrix receptors in addition to the receptors described earlier, including the EGFR and the elastin receptor, thereby promoting cell adhesion and migration in various cell types. The EGF-like repeats in tenascin-C, laminin 332, thrombospondin, and secreted protein acidic and rich in cysteine protein (SPARC) can decrease cell–matrix adhesions and promote cell migration [12]. Although receptor binding and signaling induced by these matrix molecules are complicated by their ability to bind and activate integrins, there is compelling evidence that the EGF-like repeats of tenascin C and laminin 332 promote changes in cell adhesion and motility in an EGFR-dependent manner [12]. Another matrix fragment-binding receptor, the ERC, binds fragments of elastin, laminin, and fibrillin. Although ERC binding to ligands initially promotes cell adhesion by promoting elastin deposition into the ECM, interaction of the EBP component of the ERC with elastin-derived peptides can promote migration of multiple cell types, including monocytes, keratinocytes, fibroblasts, smooth muscle cells, and endothelial cells [50]. The proliferative and migratory effects of elastin-derived peptides may result from the activation of multiple MAPK cascades downstream of the ERC [51].

In contrast to the ERC and EGFR, which can bind matrix fragments, DDR1 and DDR2 bind intact collagen molecules rather than fragments [25]. Signaling induced by collagen through the DDRs seem to have cell type–specific effects, promoting migration in some cell types while repressing it in others [25]. In the case of DDR2, collagen binding promotes the movement of fibroblasts through matrix, likely through induction of matrix metalloproteinase (MMP)-2 expression and activation, suggests a role in tissue invasion [26]. Because collagen induces relatively long-term DDR2 activation, DDR2-induced cell behaviors, such as matrix invasion, could be maintained over substantial periods [26].

Proliferation and Survival

ECM interaction with its receptors can promote cell proliferation and survival, as is demonstrated clearly by the anchorage dependence of cell growth. Even in the presence of growth factors, cells will not enter the S phase of the cell cycle without matrix adhesion [52]. In addition, cell detachment from the matrix or expression of matrix receptors in the absence of their intact ligands often promotes apoptosis, a process known as anoikis [15]. Thus, adhesion of cells to ECM molecules has an important role in regulating cell survival and proliferation. Because much of our understanding of matrix-induced proliferation and survival is related to matrix interaction with integrins, we will begin with integrin-associated proliferation and survival.

Multiple studies in which integrins are either inhibited or deficient demonstrate that integrin signaling is critical for cell proliferation [52]. For example, the fibroblasts of mice lacking the $\alpha_1\beta_1$ integrin, a primary collagen receptor, have reduced proliferation even though they exhibit normal adhesion [53]. The loss of integrin-mediated adhesion inhibits cell survival by inducing the movement of the proapoptotic protein Bax from the cytoplasm to the mitochondria, promoting apoptosis [54]. Integrin-mediated, adhesion-induced cell survival requires FAK activity, as shown by the Bax translocation and apoptosis of cells expressing dominant negative FAK and the survival of detached cancer cells overexpressing FAK [28,54]. Integrin–matrix binding activates the FAK–Src complex, which interacts with and activates PI3K, leading to the downstream activation of Akt [52]. Akt then alters the ratios of proapoptotic and antiapoptotic Bcl-2 family members, increasing the levels of the antiapoptotic Bcl-xl and Mcl-1 and decreasing the levels of proapoptotic Bax and Bak, thereby promoting cell survival [55]. Whereas the prosurvival signals downstream of Fak in epithelial cells require Src activity, as described previously, prosurvival signaling in fibroblasts instead involves p130Cas activation, which suggests that the mechanisms involved in FAK-induced survival are cell type specific [56].

Although maintenance of cell survival by integrin-mediated matrix adhesion is necessary for cell division, it is not sufficient. Many signaling pathways induced by integrin-ECM binding, notably the activation of MAPK pathways, promote cell division rather than simply survival [52]. As mentioned in an earlier section, FAK-induced p130Cas activation is upstream of Rac1, and once Rac1 is activated by this pathway, it can promote cell proliferation downstream of multiple effectors [28]. For example, Rac1-induced JNK activity promotes cell division through c-Jun-induced cyclin expression, whereas Rac1-mediated activation of Pak1 induces proliferation through its phosphorylation of Raf and downstream activation of extracellular signal-regulated kinase (ERK) [52]. Integrin ligation also activates ERK1/2 through FAK-mediated recruitment of Shc, an adaptor protein that binds Grb2/Sos and induces the Ras/ERK cascade [57]. Furthermore, cell-ECM binding and Rac activation promote the degradation of cyclin-dependent kinase (CDK) inhibitors, which would otherwise block cell proliferation [52].

Matrix molecules can also induce cell proliferation through cooperation with growth factor receptor signaling [8]. Such cooperative effects may occur in a direct manner, because some matrix molecules bind and activate growth factor receptors, leading to cell proliferation, whereas other matrix molecules bind growth factors and regulate their interaction with their receptors [23]. The matrix molecules that bind growth factor receptors directly can promote receptor activation and downstream signaling. For example, laminin and tenascin-C can bind and activate EGFR and associated downstream signaling through their EGF-like domains [12], which suggests the possibility that they or their proteolytic fragments may promote cell division downstream of EGFR. In contrast, decorin binding to EGFR and VEGFR2 inhibits proliferation induced by VEGF and EGF, potentially through internalization or degradation of the receptors, which has been shown for EGFR [12,58].

Although there is some evidence, described earlier, that matrix–growth factor receptor engagement can promote cell proliferation, substantially more evidence demonstrates the importance of growth factor binding to matrix molecules on growth factor receptor activation and induction of cell proliferation. Multiple ECM molecules are able to bind to either growth factors or their receptors to regulate their activity [8]. For example, VEGF binding to vitronectin promotes the formation of a VEGFR2/integrin $\alpha_v\beta_3$ complex that greatly increases VEGF-induced VEGFR2

phosphorylation and endothelial cell proliferation, and the binding of VEGF to fibronectin has a similar effect on VEGFR2/integrin $\alpha_5\beta_1$ complex formation, VEGFR2 activation, and cell proliferation [43,59]. Several growth factors, including FGFs, VEGFs, PDGFs, and TGFβ, can interact with HSPGs, which can either sequester these factors within the matrix, such that they are released upon matrix degradation, or can present them to their receptors [16]. For example, FGF binding to heparan sulfate moieties on syndecans facilitates FGF binding to and activation of FGFR, increasing the duration of signaling downstream of FGFR and promoting cell proliferation [16,45]. Similarly, VEGF binding to HSPGs increases binding to VEGFR2 and promotes cell proliferation [45].

In addition to matrix binding to growth factor receptors and growth factor binding to matrix, matrix–growth factor cooperation can occur through direct interactions between integrins and growth factors or growth factor receptors. VEGF binds directly to integrin $\alpha_9\beta_1$, which is necessary for VEGF-induced angiogenesis in vivo [59]. Other growth factors are also able to bind integrins directly; for example, insulin-like growth factor (IGF)-1 and FGF-2 both interact with integrin $\alpha_v\beta_3$, which is necessary for the cell proliferation induced by both factors [57]. Growth factor receptor binding to integrins may also enhance growth factor–induced signaling. Both PDGFRβ and VEGFR2 physically interact with integrin subunits, and concomitant integrin-mediated cell adhesion further increases both receptor activation and mitogenicity [52,59]. In the case of VEGFR2, integrin binding participates in FAK-induced Ras/ERK activation in endothelial cells, providing a likely mechanism for the role of integrin binding in proliferation [60].

Nonintegrin ECM receptors, including the hyaluronan receptor CD44, the ERC, and the collagen-binding DDRs, have also been implicated in cell proliferation and survival. Whereas high–molecular weight hyaluronan inhibits cell proliferation, the lower–molecular weight forms present in sites of injury and inflammation induce proliferation of multiple cell types, including fibroblasts and smooth muscle cells [19,47]. Hyaluronan-induced proliferation in fibroblasts appears to be mediated, at least in part, by CD44 and its downstream activation of ERK and Akt [61]. Similarly, binding of hyaluronan fragments to CD44 promotes smooth muscle cell proliferation via downstream activation of the Ras–ERK pathway [47]. The binding of elastin-derived peptides to the ERC promotes smooth muscle cell proliferation by activating multiple signaling pathways that culminate in activation of the MAPK cascade and upregulation of multiple cyclins and CDKs [62]. The ERC may also inhibit proliferation induced by growth factor receptors, because the neuraminidase subunit of the ERC desialylates cell surface growth factor receptors, preventing growth factor–receptor binding and downstream signaling, complicating the role that the ERC has in regulating proliferation [63]. Finally, both DDR2, a collagen receptor, and annexin II, a tenascin C receptor, can promote division in some cell types [23,26]. To date, we have few clues regarding signaling activated in matrix-induced proliferation via nonintegrin receptors, which suggests the need for more research in this area.

Differentiation

Interaction of cells with ECM molecules, hormones, and growth factors is required to activate genes needed for the differentiation of multiple cell types, including keratinocytes, endothelial cells, and fibroblasts. Differentiation of keratinocytes is carefully regulated by multiple cell–matrix and cell–cell interactions. Keratinocytes in contact with the basement membrane proliferate and do not express proteins associated with terminal differentiation, whereas those cells that are not attached to the basement membrane in suprabasal layers begin to express these markers of terminal differentiation. This suggests that keratinocyte differentiation is repressed by adhesion to the basement membrane. In support of this idea, integrin activation in cultured cells inhibits differentiation, whereas impaired adhesion promotes differentiation [64]. In contrast, keratinocytes of mice deficient in various integrins can still differentiate, which suggests that there may be some redundancy in the system [65]. There may be additional cues provided by matrix molecules present within the epidermis itself that regulate differentiation. For example, hyaluronan is present within the epidermis, where it facilitates keratinocyte terminal differentiation in a CD44-dependent manner [66]. These results are contradicted by separate studies suggesting that epidermal hyaluronan prevents premature keratinocyte differentiation [18]. One potential explanation for this discrepancy is that enzymatic digestion of hyaluronan may have generated hyaluronan fragments with binding and/or signaling differences from intact hyaluronan. Regardless, it appears that hyaluronan and CD44 are involved in the keratinocyte differentiation process.

Other differentiated phenotypes also require integrin-mediated signaling events. TGFβ-mediated differentiation of fibroblasts into myofibroblasts, contractile cells that produce ECM, requires cell integrin-mediated adhesion to extra domain A (EDA) of fibronectin [27]. Integrins $\alpha_v\beta_5$ and $\alpha_v\beta_6$ and integrin heterodimers containing the β_1 subunit are associated with TGFβ activation and are thus important in myofibroblast differentiation

[27,65]. Because integrin $\alpha_5\beta_1$ interacts with the EDA of fibronectin and integrin β_1-deficient mice exhibit defective myofibroblast differentiation, the connection between integrin $\alpha_5\beta_1$ and the fibronectin EDA may be particularly important in myofibroblast differentiation [27]. Pathways downstream of integrin–fibronectin interaction, including FAK and integrin-linked kinase activation, are necessary for TGFβ-induced myofibroblast differentiation, providing some potential mechanisms for its observed dependence on EDA-containing fibronectin [27]. Myofibroblast differentiation is also associated with matrix stiffness; increasing matrix stiffness is associated with increasing differentiation, which suggests a role for integrin-mediated mechanotransduction, likely involving FAK activation, in this differentiation process [2,5]. However, the roles of hyaluronan and CD44 activation in myofibroblast differentiation remain unclear owing to conflicting evidence in which both decreased hyaluronan production and hyaluronan-induced CD44 activation increase myofibroblast differentiation [18]. This discrepancy may result from differences in the ability of CD44 to engage in cross-talk with the TGFβ receptor under different circumstances [18].

Differentiation of endothelial cells is more difficult to identify than is differentiation of keratinocytes or myofibroblasts, both of which have specific protein markers of differentiation. In contrast, endothelial cell differentiation occurs when endothelial cells form mature vascular structures, which is challenging to mimic in vitro. Early experiments involving cultured endothelial cells demonstrated growth of endothelial cells on an ECM comparable to the basement membrane, Matrigel, induced the formation of capillary-like tubes whose formation could serve as an in vitro proxy for endothelial cell differentiation [67]. These results suggested that the adhesion of endothelial cells with some component or components of Matrigel could promote their differentiation. The main matrix components of both Matrigel and basement membranes in blood vessels in vivo are laminins, heterotrimeric proteins composed of α, β, and γ subunits [68]. In Matrigel, the primary laminin is laminin-111 ($\alpha_1\beta_1\gamma_1$), which, in the absence of other matrix components, can promote endothelial tube formation in vitro [69]. The basement membranes of intact vessels in vivo contain laminin-411 ($\alpha_4\beta_1\gamma_1$) primarily, rather than laminin-111, and laminin-411 participates in endothelial tube formation in vitro and angiogenesis in vivo; the latter is shown by the formation of abnormal vessels in laminin α_4- and γ_1-deficient mice [68]. Peptides derived from laminins α_1 and γ_1 promote angiogenesis through integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, which suggests the potential role of laminin degradation in new vessel formation [68]. After the formation of these nascent vessels, they must be stabilized and matured by the deposition of new basement membrane and the recruitment and differentiation of pericytes, smooth muscle cells that increase endothelial barrier function and participate in depositing a new basement membrane [9,70]. Fibronectin, laminin-411, collagen IV, and HSPGs are all required for the formation of the new basement membrane, whereas integrin-mediated interactions between the developing matrix and pericytes is necessary for continued deposition of basement membrane matrix molecules [68,71]. As such, matrix–integrin interactions are critical for the deposition of the basement membrane and recruitment of pericytes, which, in turn, are necessary for new vessel maturation.

Apoptosis

Many cell types undergo apoptosis, or programmed cell death, through well-known signal transduction pathways involving the activation of proteases from the caspase family. Cell-matrix signaling tends to promote cell adhesion and survival, repressing apoptosis. However, even when cells remain attached to some ECM molecules, the inability of some integrins to bind their matrix ligands induces a specific form of apoptosis called integrin-mediated death through integrin-mediated recruitment and activation of caspase 8 [72]. In addition to promoting apoptotic signaling, the activation of caspase 3 downstream of caspase 8 may prevent prosurvival integrin signaling from separate matrix-bound integrins by cleaving paxillin and kindlin-3, blocking prosurvival outside-in signaling downstream of these proteins [37,41]. Integrin ligation by soluble, rather than intact, ligands also promotes apoptosis through the recruitment and activation of caspase 8 by the clustered integrins [73]. Such soluble ligands may be created by matrix degradation during tissue remodeling. A fragment of collagen XVIII, endostatin, binds to $\alpha_5\beta_1$ integrin and decreases the expression of Bcl-xl and Bcl-2, prosurvival Bcl family members, promoting endothelial cell apoptosis [10]. Similarly, a fragment of collagen IV, tumstatin, induces apoptosis in endothelial cells via integrin $\alpha_v\beta_3$ [74]. Fragments of matrix molecules can also induce apoptosis through nonintegrin receptors. Low–molecular weight fragments of hyaluronan can induce apoptosis in some cells through CD44, likely by interfering with prosurvival signaling downstream of CD44 [19]. Similarly, elastin-derived fragments promote fibroblast and lymphocyte apoptosis through the ERC [22,51]. Taken together, these pieces of evidence suggest that disrupting normal matrix–receptor adhesions can promote apoptosis in various cell types.

CELL–EXTRACELLULAR MATRIX INTERACTIONS DURING HEALING OF CUTANEOUS WOUNDS

Interactions of cells with ECM molecules have a crucial role during wound healing and regeneration. Continuous cross-talk between cells and the surrounding matrix environment contributes to the processes of clot formation, inflammation, granulation tissue development, and remodeling; during regeneration, matrix interactions are important in restoring damaged tissue. Many lines of experimental evidence demonstrate that the basic cellular mechanisms resulting in wound healing involve cell adhesion–deadhesion, migration, proliferation, differentiation, and apoptosis (Fig. 2.2).

Adhesion and Migration

Shortly after tissue damage and during the early stages of wound healing, multiple factors and blood cells enter into the wound area, activating the coagulation cascade when coagulation factors from the blood encounter tissue factor expressed on endothelial cells, tissue factor expressed by nonvascular cells exposed by injury, or collagen, also exposed by injury [75]. This cascade ultimately results in the activation of thrombin, an enzyme that cleaves fibrinogen to generate fibrin, which polymerizes to form a fibrin clot. Injury to the endothelium simultaneously promotes the adhesion of platelets to subendothelial von Willebrand Factor and ECM components, causing platelet aggregation, activation, and adhesion to fibrin, trapping them within the fibrin clot [75]. Activated platelets release additional coagulation factors that promote and stabilize the fibrin clot, which serves as a vascular plug. In addition, to fibrin and platelets, the fibrin clot contains matrix components including plasma fibronectin and a variety of chemokines, cytokines, and growth factors released by activated platelets [7,76]. As such, in addition to its hemostatic function, the fibrin clot facilitates wound healing by serving as a provisional matrix for cell migration and a reservoir of cytokines, thrombin, and growth factors that collectively promote the later phases of inflammation and granulation tissue formation [77].

During the clotting process, platelets and activated mast cells degranulate, releasing vasodilating and chemotactic factors that chemoattract inflammatory leukocytes to the wound site, initiating the inflammatory response. Leukocyte extravasation from blood vessels requires several adhesion and signaling events, including the binding of leukocyte integrins with endothelial intercellular cell adhesion molecules and vascular cell adhesion molecules rather than matrix molecules, and the binding of leukocyte chemokine receptors with chemokines associated with endothelial cell surface HSPGs [27,78]. Some of the first matrix molecules that the leukocytes encounter during emigration from the bloodstream are located within the endothelial basement membrane and the provisional matrix. Neutrophils adhere to and migrate within the basement membrane as they move through the blood vessel, where they encounter laminins-411 and -511, fibronectin, and vitronectin, and subsequently interact with fibrin and fibronectin in the provisional matrix [76,79]. Neutrophils also secrete laminin-411, which likely participates in their extravasation through integrin $\alpha_M\beta_2$, because inhibition of this integrin blocks leukocyte extravasation [79].

After leukocyte extravasation from the vasculature, the leukocytes are directed to the site of injury by the chemokines that form relatively stable gradients through interactions with endothelial cell surface proteins and ECM molecules, thereby promoting directional cell migration through the provisional matrix [6]. The fibrin–fibronectin provisional matrix serves as substrate for the migration of leukocytes and later keratinocytes during the early stages of healing when inflammation and reepithelialization occur. Leukocyte interactions with ECM molecules via integrin receptors affect many of the functions of these cells, in particular those associated with cell adhesion, migration, the production of inflammatory mediators, and antimicrobial functions. As mentioned, neutrophils interact with fibronectin in the basement membrane and the provisional matrix, and several types of inflammatory cells interact with fibrinogen, the primary component of the provisional matrix, through integrins $\alpha_M\beta_2$ and integrin $\alpha_X\beta_2$ [77,80]. Neutrophil binding to thrombospondin 4, another ligand of integrin $\alpha_M\beta_2$, also induces expression of the chemokine CXCL8, whereas monocyte interactions with fibrin in the provisional matrix induces expression of multiple proinflammatory cytokines and chemokines, including TNFα, interleukin (IL)-6, IL-1β, and several CC chemokines [80,81]. Intact ECM molecules then facilitate additional leukocyte chemotaxis into the inflamed area by binding these chemokines, thus creating a stable chemotactic gradient to promote specific directional migration [7,82].

Although intact ECM molecules regulate inflammation in multiple ways, matrix fragments also participate in this process. Inflamed tissues contain many proteases that can cleave matrix molecules, generating fragments with

altered matrix binding, downstream signaling, and effects on inflammatory processes. For example, platelet-derived hyaluronidase cleaves hyaluronan, generating low–molecular weight hyaluronan fragments; neutrophil elastase cleaves elastin, fibronectin, and collagen XVII; and urinary plasminogen activator– and tissue plasminogen activator–induced plasmin activation promote cleavage of collagen XIX, laminin-332, SPARC, and syndecan-4 [4,6,47]. Several of the fragments generated in this manner then promote inflammation in a positive feedback loop. For example, low–molecular weight hyaluronan fragments induce cytokine and chemokine production through CD44, and collagen XVII and elastin fragments generated by neutrophil elastase promote neutrophil chemotaxis [4,19]. Ultimately, the matrix molecules encountered by leukocytes substantially influence the course of inflammation.

The inflammatory phase of wound healing is followed by a proliferative phase. Shortly after wounding, activated platelets secrete a variety of growth factors, including EGF and TGFβ, that stimulate the keratinocytes at the wound edge to proliferate and migrate to cover the wounded area, a process known as reepithelialization [83]. FGFs and EGFs are produced and/or released from sequestering matrix molecules at later times by neutrophils, macrophages, endothelial cells, and fibroblasts, and may maintain the proliferative and promigratory signals needed for reepithelialization. During reepithelialization, the keratinocytes migrate beneath the provisional ECM, composed primarily of fibrin and fibronectin [27,65]. The lack of keratinocyte migration on top of the fibrin-based clot may result from their lack of integrin $\alpha_v\beta_3$ expression as well as the ability of fibrin to prevent keratinocyte adhesion to other provisional matrix components, including fibronectin [77]. These cells instead migrate on the nascent, provisional basement membrane composed largely of including laminin-332, fibronectin, and tenascin-C, through the $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_9\beta_1$, $\alpha_2\beta_4$, and α_v integrins expressed by these cells [27]. That these matrix–receptor interactions are critical for reepithelialization is clear from studies investigating keratinocyte migration and reepithelialization in cultured keratinocytes migrating on specific matrices, in mice lacking these molecules, and in human patients with matrix mutations. Interestingly, keratinocytes at the migration front produce laminin-332, facilitating the migration of keratinocytes behind the migration front on this matrix component [27]. Because the keratinocytes migrate between the fibrin-based clot and the underlying tissue, their migration is also associated with the activity of multiple proteases, including MMPs and plasmin [27,84]. These enzymes may facilitate keratinocyte migration by promoting their deadhesion from matrix molecules that would otherwise promote adhesion over migration and/or through releasing matrix-bound growth factors, or EGF-like domains from matrix molecules themselves, that then induce promigratory signaling [65,84].

At the same time as reepithelialization, granulation tissue, a provisional connective tissue containing nascent blood vessels and multiple types of ECM molecules, including tenascin-C, cellular fibronectin, SPARC, and various collagens, begins to form [7,26,27]. Granulation tissue serves as substrate for the migration of the keratinocytes (see earlier discussion), the endothelial cells that form the vasculature of the wound bed, fibroblasts, myofibroblasts, and leukocytes that are chemoattracted to the wound site by chemokines secreted by multiple cells within the wound [26]. Chemokine-mediated chemoattraction of cells involved in granulated tissue formation, in conjunction with the interaction of these cells with ECM via cell surface receptors, participates in functions during the formation of the granulation tissue. For example, the neutrophil chemoattractant CXCL8, produced by multiple cell types in the wound environment, induces fibroblast recruitment to the granulation tissue and their deposition of tenascin-C, fibronectin, and collagen I [85].

Interactions of both intact matrix molecules and their degradation products with endothelial cells facilitate their migration through the nascent connective tissue to generate new blood vessels during the formation of granulation tissue [27]. As described in more detail previously, fibronectin and vitronectin may promote endothelial cell migration and angiogenesis by enhancing VEGF-induced promigratory signaling through the formation of integrin–VEGFR2–VEGF–matrix molecule complexes [59]. Proteoglycans may promote endothelial cell migration and angiogenesis through their association with angiogenic factors, including VEGF, CXCL8, and FGF-2, generating a stable gradient to promote directional migration and vessel formation [9,22,85]. During angiogenesis, endothelial cells release and activate matrix-degrading enzymes, including MMPs and cathepsins, which can facilitate the migration and invasion of endothelial cells into the surrounding tissue and generate bioactive matrix fragments that provide additional angiogenic stimuli [4]. Complicating the role of matrix fragments in angiogenesis is that some matrix fragments promote angiogenesis whereas others inhibit angiogenesis by inhibiting endothelial cell migration and/or inducing their apoptosis [4]. This raises the possibility that after angiogenic matrix molecules and fragments induce angiogenesis, these molecules could be cleaved further to generate antiangiogenic fragments that then promote vessel stabilization, basement membrane deposition, and maturation [86]. Therefore, the way matrix molecules are locally cleaved and/or factors are locally released could have important consequences for the formation of the granulation tissue.

Proliferation

During wound reepithelialization, keratinocytes trailing behind those at the front edge of migration replicate to provide a source of cells that cover the wound. Basement membrane-type ECM still present on the basal surface of these keratinocytes may be important in maintaining this less migratory, proliferative state. In a dermal wound model, basement membrane matrices are able to sustain the proliferation of keratinocytes for several days [87]. One component of the basement membrane involved in this proliferation is likely laminin. For example, laminin-511 and -521 can promote keratinocyte proliferation in vitro, and keratinocyte proliferation requires the laminin receptor integrin $\alpha_6\beta_4$ [52,68]. Integrin $\alpha_9\beta_1$, which binds several matrix molecules, is critical for keratinocyte proliferation during wound healing and thus in reepithelialization [65]. In contrast, specific cell–matrix interactions may prevent excessive proliferation. For example, the keratinocytes of mice deficient in either fibrinogen or emilin-1, a matrix ligand for integrins $\alpha_4\beta_1$ and $\alpha_9\beta_1$, hyperproliferate during reepithelialization, which suggests the importance of these molecules in limiting proliferation during normal reepithelialization [27,52].

As described earlier, granulation tissue forms at the same time as reepithelialization. In the granulation tissue, several types of cells proliferate, including fibroblasts and endothelial cells of the microvasculature. The ECM molecules present in the granulation tissue, in conjunction with growth factors released by the platelets and secreted by the cells present in this tissue, provide signals to promote cell proliferation [8]. ECM molecules themselves, including fibronectin and specific fragments of fibronectin, tenascin-C, laminins, collagen VI, SPARC, and hyaluronan, can stimulate fibroblast and endothelial cell proliferation [2]. For example, fibroblast proliferation requires collagen-induced activation of DDR2, a tyrosine kinase receptor, as shown by the reduced proliferation of DDR2$^{-/-}$ in vitro and decreased numbers in wound granulation tissue in vivo, although the decreased numbers in vivo could result from a combination of decreased proliferation and migration [26]. ECM molecules may cooperate with growth factors to promote fibroblast and endothelial cell proliferation. In fibroblasts, proliferation induced by TGFβ1 requires fibronectin [88]. Angiogenesis requires both endothelial cell migration (described previously) and proliferation, and angiogenic factors such as VEGFs and FGFs associate with ECM molecules, increasing their ability to activate their receptors and thereby stimulate the proliferation of endothelial cells, which then migrate to form the new microvessels [9,59,85]. Some antiangiogenic molecules, including thrombospondin and endostatin, may inhibit angiogenesis by competing with these growth factors for either growth factor receptor binding or matrix binding [9,10]. In contrast, ECM molecules and/or peptides derived from their proteolysis can have inhibitory effects on cell proliferation. Intact decorin and SPARC, as well as peptides derived from decorin, SPARC, collagens XVIII and XV (endostatin), collagen IV (tumstatin), and tenascin-C have antiangiogenic effects owing to their inhibition of endothelial cell proliferation [4,22].

Differentiation

As healing progresses, the healing wound shifts from granulation tissue formation to matrix remodeling, gradually removing the provisional matrix molecules of the granulation tissue and replacing them with a more mature connective tissue rich in collagen I [2]. This process is associated with the differentiation of some fibroblasts into myofibroblasts, acquiring the morphological and biochemical characteristics of smooth muscle cells by expressing α-smooth muscle actin [84]. This differentiation process requires specific growth factors and/or chemokines, including TGFβ and CXCL8, as well as fibroblast interactions with multiple types of matrix molecules [84,85]. TGFβ1-induced myofibroblast differentiation requires adhesion to the EDA-containing splice variant of fibronectin, likely mediated by integrin $\alpha_4\beta_7$ [5,89]. However, the combined presence of EDA-containing fibronectin and TGFβ1 is not sufficient to induce myofibroblast differentiation, which also requires fibroblast adhesion to stiff collagen matrices [2]. After myofibroblast differentiation, these cells secrete copious amounts of matrix molecules, particularly multiple isoforms of collagens, release enzymes that cross-link and thereby stiffen collagen fibrils further, and contract to promote wound closure [26,84]. These myofibroblast activities could cause more fibroblasts to differentiate into myofibroblasts in a positive feedback loop that, if unchecked, could promote excessive fibrosis and abnormal scarring that interferes with normal tissue function [84]. Decorin, a small proteoglycan present in normal wound healing, decreases TGFβ1 and collagen production, providing a possible mechanism that could limit myofibroblast differentiation and function to prevent excessive scarring [84].

Differentiation of keratinocytes, endothelial cells, and pericytes is also regulated by cell–matrix interactions. In keratinocytes, some matrix–integrin interactions seem to inhibit terminal differentiation and promote proliferation of basement membrane–associated cells, whereas hyaluronan–CD44 binding may promote terminal differentiation [64,66]. The differentiation of endothelial cells in mature blood vessels requires physical interactions with basement

membrane components such as laminin-411, as well as interactions with pericytes [68]. Pericyte differentiation, in turn, requires integrin β_1–dependent cell–matrix interactions [90].

Apoptosis

In healing wounds, many cells that are needed for one specific phase of the healing process undergo apoptosis after completing their respective functions. In fact, the persistence of some cells, including inflammatory cells and myofibroblasts, is detrimental, and apoptosis is needed to prevent chronic inflammation and excessive scarring, respectively [89,91]. ECM molecules regulate some of the inflammatory cell apoptosis after their activation. For example, low–molecular weight hyaluronan promotes neutrophil and macrophage apoptosis, likely through CD44 [19,47]. However, apoptosis in neutrophils is primarily regulated by a constitutive signaling pathway that can be delayed by inflammatory signaling or by fibrinogen binding, but otherwise seems little affected by matrix interactions [91,92].

Apoptosis also participates in the wound remodeling phase, because the granulation tissue evolves into a relatively acellular scar tissue [89]. In this remodeling phase, apoptotic cell death eliminates many types of cells at the same time without causing tissue damage. Within the granulation tissue, the number of cells undergoing apoptosis increases around days 20–25 after injury, dramatically reducing wound cellularity after day 25 [89a]. This coincides with the release of mechanical tension after myofibroblast-mediated wound contraction, which triggers apoptosis of human fibroblasts and myofibroblasts, which suggests the importance of interstitial collagens, their receptors, and mechanotransduction in myofibroblast apoptosis [7]. This myofibroblast apoptosis may be required to both promote wound healing and prevent scarring. Indeed, a type of excessive, abnormal scar called a hypertrophic scar exhibits reduced fibroblast/myofibroblast apoptosis, resulting in excessive fibrosis and scarring [84]. This apoptotic failure in hypertrophic scars likely results from an overexpression of tissue transglutaminase, leading to increased matrix breakdown and decreased collagen contraction [2].

CELL–EXTRACELLULAR MATRIX INTERACTIONS DURING REGENERATIVE FETAL WOUND HEALING

True tissue regeneration after injury rarely occurs in vertebrate species, but it occurs in specific instances, including fetal cutaneous wound healing, liver regeneration, and urodele amphibian limb regeneration. Unlike wound healing in normal adult animals, which is characterized by scarring, fetal cutaneous wounds heal without fibrosis and scar formation, leading to regeneration of the injured area. The contribution of cell–ECM interactions to regeneration in fetal healing is discussed subsequently (Fig. 2.3).

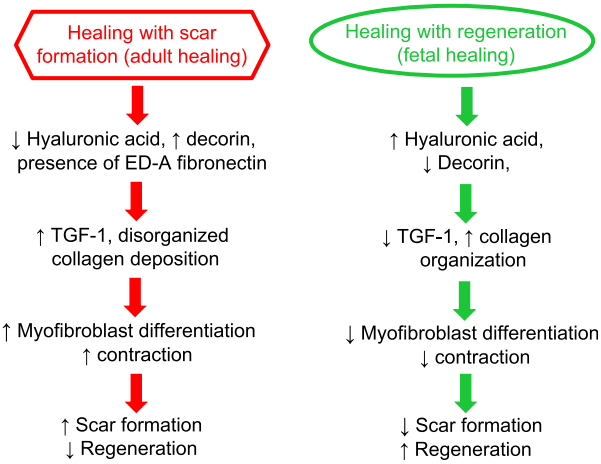

FIGURE 2.3 Comparison of particular cell–extracellular matrix (ECM) interactions occurring in scar-forming adult healing versus those occurring during regenerative fetal healing. As shown in this diagram, unique subsets of ECM molecules are associated with scarring versus regenerative healing. As such, therapeutic alteration of ECM composition may allow physicians to modulate healing to promote tissue regeneration. Additional therapeutic approaches may be generated upon further investigation into the importance of additional cell–ECM interactions in scarring and regenerative responses. *ED-A*, extra domain A; *TGF-1*, transforming growth factor-1.

Adhesion and Migration

Fetal wounds, at least these relatively early in fetal development, heal without scarring, in contrast to most adult healing, which heals with at least some scarring [89]. One major difference between scarless fetal healing and adult healing is the lack of an inflammatory response before embryonic day (E)15–16 in mouse development, and the attenuated inflammatory response seen after E18 [7]. Indeed, the onset of scarring in fetal healing at later embryonic stages coincides with the substantial appearance of mast cells and neutrophils in fetal wounds [2,93,94]. Mast cells after E18 have much larger mast cell granules, which contain higher levels of proinflammatory cytokines that may then contribute to the increased inflammatory response after this stage of development [95]. This lack of neutrophils in early fetal wounds is also associated with the low expression of neutrophil adhesion molecules, decreasing extravasation coupled with low levels proinflammatory cytokines chemokines such as TNFα, IL-6, and CXCL8, and increased levels of antiinflammatory cytokines such as IL-10 [96]. Experimental increases in IL-10 in adult wounds reduced inflammation and promoted regenerative healing, providing important evidence for the role of inflammation in the neutrophil recruitment and scarring present in adult wounds [89,96].

Scarless fetal wounds also differ from scarring adult wounds in cell–ECM interactions owing to differences in the composition of the ECM molecules, the timing of their appearance after wounding, and their duration in the wound area. One crucial ECM molecule in fetal wound healing is hyaluronan, which appears to be necessary for the regenerative response, because its removal from fetal wounds promotes a healing response more similar to that of adults, and treatment of normally scarring wounds or wound organ cultures with hyaluronan decreases scarring [97–99]. High–molecular weight hyaluronan is more abundant in fetal skin wounds than in adult wounds, where low–molecular weight hyaluronan is more abundant; the latter possibly results from increased hyaluronidase activity in adult wounds [2,83]. Fetal fibroblasts also express higher levels of the hyaluronan receptors CD44 and RHAMM after injury, thus increasing receptor–ligand interactions that promote fibroblast migration [98].

Tenascin C, fibronectin, and collagen levels also differ in adult and fetal wounds. Tenascin C is expressed at higher levels in fetal skin than in adult skin and is induced more rapidly and to a greater extent in fetal wounds, modulating cell adhesion to fibronectin and promoting migration within matrices containing fibronectin [27]. Fibronectin production increases more rapidly in fetal wounds, although the fibronectin produced does not contain the profibrotic EDA domains [2,27]. This increased expression of tenascin and fibronectin is associated with concomitant increases in the expression of integrins that serve as their receptors. In particular, α_5 subunit, $\alpha_v\beta_3$, and $\alpha_v\beta_6$ integrins, which bind fibronectin and/or tenascin, are upregulated in the wounded fetal epithelium [100]. The combined rapid increases in fibronectin and tenascin, coupled with increased expression of their respective integrin receptors in epithelial cells, are likely important in facilitating cell migration and reepithelialization in fetal wounds. In contrast to the increased levels and/or rate of tenascin C and fibronectin production in fetal wounds, these wounds contain reduced levels of total collagen compared with adult wounds. However, fetal wounds contain a greater proportion of collagen III compared with collagen I than do their adult counterparts [84]. The observed changes in the collagen I/III ratio in fetal wounds and their relative lack of collagen deposition and fibrosis may result from changes in the deposition, organization, and cross-linking of collagen at the wound site or rapid turnover of these ECM components by protease-mediated degradation [84,98]. Related to increases in matrix turnover, fetal wounds have increased levels and activity of multiple MMPs, with decreased levels of their endogenous inhibitors, tissue inhibitor of metalloproteinases, ultimately promoting matrix degradation and turnover [84]. Not only does the resulting matrix degradation and turnover prevent fibrosis, it also likely facilitates cell migration by reducing matrix density and increases the generation of proteolytic matrix fragments that modulate various stages of wound repair.

Proliferation

Increased levels of hyaluronan present during fetal wound healing likely decrease fetal fibroblast proliferation [101]. Fetal fibroblasts also exhibit decreased proliferation in response to growth factors compared with that of adult cells. For example, IGF-1, which induces ERK signaling, proliferation, and matrix synthesis in postnatal fibroblasts, induces proliferation to a much lesser extent and fails to induce significant ERK signaling or matrix synthesis in fetal fibroblasts [102]. Furthermore, whereas TGFβ1 induces proliferation in postnatal fibroblasts, it does not do so in fetal fibroblasts, possibly because of the ability of TGFβ1 to induce hyaluronan synthesis in fetal but not postnatal fibroblasts [103].

Differentiation

Fetal wounds have a decreased number of myofibroblasts, which appear in the wounded site earlier and remain a shorter time than in adult wounds [89,98]. Fetal fibroblasts produce more type III collagen and less type I collagen than adult cells, and the diameter and organization of the fibrils in the fetal wound are comparable to unwounded skin, whereas those of the adult wound exhibit a disorganization indicative of scarring [98,104]. Adult wound tissue is stiffer than fetal tissue, likely resulting from increased collagen I levels in adult wounds, facilitating myofibroblast differentiation that depends on stiff collagen matrices [2]. In contrast, fetal wounds have a decreased number of myofibroblasts, which appear in the wounded site earlier and are more transient than in adult wounds [89,98]. The low number of myofibroblasts in fetal wounds may result, at least in part, from a lack of collagen matrix stiffness [83]. Because myofibroblasts are themselves responsible for much of the collagen I production and tissue contraction in adult wound tissue, their relative absence in fetal wounds may be responsible for the reduced collagen I levels and lower contraction in fetal wounds [89].

Increased fetal hyaluronan may also prevent myofibroblast differentiation by increasing expression of TGFβ3, which is antifibrotic, in contrast to TGFβ1, which increases collagen I deposition and promotes scar formation [84,98]. Indeed, adult wounds treated with hyaluronan healed more rapidly with a significant decrease in TGFβ1 levels, which suggests that the large amounts of hyaluronan in fetal wounds may thus explain, at least partly, the greatly reduced levels of TGFβ1 in fetal wounds [83]. Downregulation of TGFβ1 in adult wounds produces a decrease in scarring similar to that observed with hyaluronan treatment, whereas addition of TGFβ1 to normally scarless fetal wounds induces a more scarring phenotype, with myofibroblast differentiation, wound contraction, and fibrosis [98,105]. Thus, hyaluronan-mediated inhibition of TGFβ1 expression may be critical in scarless fetal healing. The relatively small amount of TGFβ1 present during fetal wound healing may be regulated by inhibitory ECM molecules present in the injured area. One such inhibitor is the proteoglycan decorin, which is capable of binding TGFβ1 and preventing receptor activation and is expressed to a greater extent in fetal wounds than in adult wounds [2]. Decreased activity of this growth factor, combined with low levels of expression in fetal wounds, likely results in decreased fibrosis, myofibroblast differentiation, and wound contraction, leading to regeneration rather than scarring.

Apoptosis

Less is known about the apoptotic process in fetal wounds than in adult wounds, which makes it difficult to compare cellular apoptosis under these conditions. An investigation of apoptotic induction at very early time points after wounding in both scarless (E15) and scar-forming (E18) fetal mouse wounds found lower apoptosis in scar-forming wounds than scarless wounds [106]. Some of these cells disappearing from scarless wounds may be myofibroblasts, because any myofibroblasts that differentiate from fetal fibroblasts, either in vivo or in vitro, disappear rapidly, perhaps owing to an altered rate of apoptosis in these wounds [106]. If changes in apoptotic efficiency indeed occur, they may result from the decreased contraction, and thus decreased mechanical tension, in fetal wounds, as well as altered collagen levels within the collagen matrix [7]. However, myofibroblasts may disappear from fetal wounds through dedifferentiation back to fibroblasts, which complicates the picture [89]. Perhaps apoptosis is not as critical in the healing of fetal wounds as in adult wounds, because leukocyte influx and myofibroblast differentiation appear to be minimal in fetal wounds, and thus may not require large numbers of cells to undergo apoptosis for regeneration to occur [7,89].

IMPLICATIONS FOR REGENERATIVE MEDICINE

One primary goal of studies comparing differences in cell–ECM interactions, and thus changes in signaling, that accompany regenerative and nonregenerative healing is to determine which types of interactions promote and which inhibit tissue regeneration (for an example, see Fig. 2.3). After elucidating the functions of particular interactions, it may be possible to increase the regenerative response through (1) the induction of proregenerative ECM molecules or signaling events in the wounded area combined with (2) the antagonism of antiregenerative–scarring interactions or signaling events using specific inhibitors. This discussion of regenerative medicine will focus on possible strategies to promote regeneration in adult scarring wounds, thus causing adult wounds to resemble more closely fetal scarless wounds. Such an increased regenerative response would be particularly useful in treating wounds that heal with increased scar formation, such as keloids and hypertrophic scars.

Different types of approaches may be used to increase proregenerative ECM levels in the wounded area, including direct application of the matrix molecules themselves, the addition of agents that increase their expression, the addition of cells producing these types of ECM that have been prepared to minimize immunogenicity, the introduction of biomaterials modified to contain adhesive, proregenerative regions of these ECM molecules, or wound treatment with inhibitors of their proteolysis.

Several different ECM molecules are present at higher levels in fetal wounds than in adult wounds, including hyaluronan, tenascin, fibronectin, and collagen III, which may have important roles in the regeneration process [2,27,98]. Thus, altering the levels of these molecules in a scarring wound may improve regeneration. In keeping with the substantial evidence supporting a role for hyaluronan in scarless healing, multiple types of biomaterials currently used to promote healing incorporate hyaluronan in the presence or absence of other matrix molecules or various cell types, although the antiscarring outcomes of these biomaterials vary [107]. One potential reason for the observed variability lies in the rapid degradation of hyaluronan caused by hyaluronidase activity in vivo, and a biomaterial containing modified, more hyaluronidase-resistant hyaluronan mimics improved healing in multiple contexts [99]. Other biomaterials have been used with some success, including those with substantial amounts of collagen III and "natural" scaffolds containing multiple matrix molecules and proteoglycans [99,108]. Combinations of hyaluronan with other matrix molecules, including tenascin, embryonic fibronectin, and/or collagen III, should mimic the fetal wound environment better and may lead to more regenerative healing. Alternatively, various matrix scaffolds could be combined with growth factors that promote healing and reduce scarring, using the matrix molecules to deliver growth factor more effectively to the wound and thereby better facilitate a regenerative response [76]. Alterations in the biomaterial formulation, such as adding molecules that bind and release growth factors or engineering growth factors that associate more effectively with biomaterials, could regulate the timing of growth factor release, allowing their release over a relatively long period to promote more effective healing [76]. These or other biomaterials may be useful for the delivery of proregenerative ECM molecules and/or growth factors to the injured area, thereby promoting healing and reducing scar formation.

When attempting to promote regeneration, it is also imperative to inhibit events associated with scarring, including excessive ECM deposition, fibrosis, and contraction. During the adult healing process, these scar-associated processes are primarily controlled by the myofibroblast, a differentiated cell type that arises during the adult healing process, but which is largely absent throughout fetal wound healing. Therefore, inhibition of myofibroblast differentiation or function in combination with the addition of proregenerative molecules may facilitate a stronger regenerative response. Inhibition of differentiation could be accomplished by blocking factors that normally stimulate myofibroblast differentiation, such as TGFβ1 and CXCL8, by preventing fibroblast−ECM interactions that facilitate myofibroblast differentiation, such as EDA-containing fibronectin, and by the delivery of antifibrotic molecules such as TGFβ3 and IL-10 [84,85,96]. It is also possible that the application of one molecule may promote more regenerative healing by affecting multiple parts of the healing process. For example, insulin interaction with its receptor affects multiple aspects of keratinocyte behavior, stimulating cell motility, increasing expression of the cell surface adhesion molecule integrin α_3, enhancing secretion of the ECM molecule laminin-332, and improving epidermal differentiation during wound healing [109]. Furthermore, we have shown that insulin stimulates the formation of regenerative rather than scarring matrix [110].

The surge in research regarding ECM molecules themselves and their interactions with particular cells and cell surface receptors has led to the realization that such interactions are many and complex, and that they are of the utmost importance in determining cell behavior during events such as wound repair and tissue regeneration. Therefore, the manipulation of specific cell−ECM interactions has the potential to modulate particular aspects of the repair process and thereby promote a regenerative response.

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CHAPTER

3

Mechanisms of Blastema Formation and Growth in Regenerating Urodele Limbs

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INTRODUCTION

The limbs of larval and adult urodele amphibians are unique among tetrapod vertebrates in their ability to regenerate from any level of the limb after amputation. Limb regeneration can be divided into two major phases: (1) formation of a blastema that resembles the early embryonic limb bud and (2) blastema redevelopment, which involves growth and redifferentiation. Blastema formation refers in this chapter to events leading to the establishment of an accumulation of undifferentiated cells under a thickening of the distal wound epidermis, the apical epidermal cap (AEC). Pattern formation, in which the spatial relationships of the structures to be regenerated are determined and specified, is a process that spans both phases. The ability to form a blastema after amputation is what distinguishes the limbs of urodeles from those of anuran amphibians, reptiles, birds, and mammals, and is the primary focus of this chapter. Blastema formation is a reverse developmental process realized partly by cell dedifferentiation in tissues local to the amputation plane [1] and partly by a contribution of muscle stem cells [2].

Blastema development is similar to that of the embryonic limb bud, with one major exception: blastema cell proliferation depends on an interaction between the limb nerves and the AEC, whereas proliferation of embryonic limb bud cells relies on an epithelial−mesenchymal interaction among the counterpart of the AEC, the apical ectodermal ridge, and the subjacent mesenchymal cells. The musculoskeletal and skin tissues of the new limb parts derived from the blastema redifferentiate in continuity with their parent tissues, whereas blood vessels and nerves regenerate by extension from the cut ends of the preexisting blood vessels and axons, respectively. The growing blastema goes through several morphological/histological stages to attain a cone of cells that then broadens and initiates differentiation in proximal to distal and anterior to posterior directions, ending in distal bifurcations of the digits.

If we were able to understand why some animals such as urodele amphibians are able to form a regeneration-competent blastema after amputation whereas others such as adult anurans, birds, and mammals are not, it might be possible to design chemical approaches to inducing blastema formation in human appendages. At the least, such knowledge might improve our ability to deal with nonamputational injuries to musculoskeletal, vascular, and neural tissues. With this in mind, I review here what is known about blastema formation in the regeneration-competent limbs of urodeles and compare it with blastema formation in the regeneration-deficient anuran, *Xenopus laevis*.

BLASTEMA FORMATION

Blastema formation in regenerating urodele limbs can be subdivided into three phases: (1) hemostasis and reepithelialization, (2) histolysis and dedifferentiation, and (3) blastema cell migration and accumulation (Fig. 3.1). The latter two phases overlap with one another.

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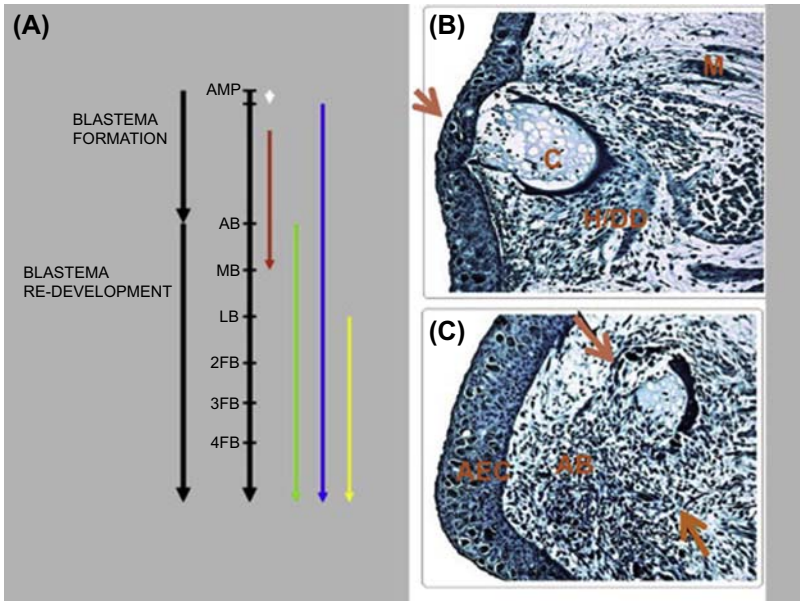

FIGURE 3.1 (A) Diagram of phases and stages of regeneration after amputation of a urodele limb. The two *black lines* indicate the two major phases of regeneration (blastema formation and blastema redevelopment) and the stages of regeneration after amputation (AMP). *2FB, 3FB, 4FB*, finger bud stages; *AB*, accumulation blastema; *LB*, late bud; *MB*, medium bud. The *colored lines* indicate different subphases of blastema formation and redevelopment. *Blue*, pattern formation; *green*, blastema growth; *orange*, histolysis and dedifferentiation; *white*, hemostasis and reepithelialization; *yellow*, redifferentiation. (B) Longitudinal section of regenerating axolotl hindlimb 4 days after amputation through the midtibia-fibula. *Arrow* points to the thickening apical epidermal cap (AEC). The cartilage (C), muscle (M), and other tissues are breaking down in a region of histolysis and dedifferentiation (H/DD) under the wound epithelium. Magnification ×10, light green and iron hematoxylin stain. (C) Longitudinal section of regenerating axolotl hindlimb 7 days after amputation through the midtibia-fibula. An accumulation blastema (AB) has formed by the migration of dedifferentiated cells under the AEC. *Arrows* mark the junction between the accumulation blastema and the still-active region of histolysis and dedifferentiation proximal to it. magnification ×10, light green and iron hematoxylin stain.

Hemostasis and Reepithelialization

After limb amputation or after making skin wounds in amphibians, vasoconstriction occurs and a thrombin-catalyzed fibrin clot forms within seconds to protect the wound tissue and provide a temporary matrix from which repair or regeneration is initiated. An epithelium two to three cells thick covers the wound surface within 24 h after amputation, depending on limb size. The basal epidermal cells at the cut edge of the skin migrate as a sheet that is extended by mitosis of cells adjacent to the wound edges [3]. The fibrin clot contains significant amounts of fibronectin, which the epithelial sheet uses as a substrate for migration [4]. Within 2–3 days postamputation (dpa), the wound epidermis thickens to form the AEC.

The basal cells and gland cells of the wound epidermis/AEC have secretory functions, as evidenced by their more extensive endoplasmic reticulum and Golgi network [5]. WE3, 4, and 6 are three secretory-related antigens expressed specifically by dermal glands and wound epidermis/AEC [6]. Two other antigens, 9G1 [7] and NvKII [8], are also specific to the wound epidermis, but their functions are unknown.

The early wound epidermis has an important function in generating early signals for limb regeneration. Na^+ influx in the amputated newt limb and H^+ efflux in the amputated tail of *Xenopus* tadpoles generate ionic currents across the wound epidermis essential for regeneration. Na^+ influx is via sodium channels [9]. H^+ efflux in the amputated tail is driven by a plasma membrane adenosine triphosphatase (ATPase) in the epidermal cells [10] and is likely to be important for urodele limb regeneration as well, given that a gene encoding a v-ATPase was the most abundant clone in a suppressive subtraction complementary DNA library made from 4-dpa regenerating limb tissue in the axolotl [11]. Drug-induced inhibition of either Na^+ or H^+ movements during the first 24 h or so after amputation results in failure of blastema formation [10,12].

Inositol triphosphate (IP_3) and diacylglycerol (DAG) are the products of phosphatidylinositol bisphosphate (PIP_2), which in turn is derived from inositol. IP_3 synthase, a key enzyme for the synthesis of inositol from glucose-6-phosphate, is upregulated during blastema formation in regenerating axolotl limbs [13]. IP_3 stimulates a rise in cytosolic Ca^{2+} that results in the localization of protein kinase C (PKC) to the plasma membrane, where PKC is activated by DAG and regulates transcription [14]. During blastema formation, there is a general

downregulation of proteins involved in Ca^{2+} homeostasis, which suggests that IP_3 might signal a rise in cytosolic Ca^{2+} in regenerating limbs to localize PKC to the plasma membrane [13]. Other studies have shown that IP_3 is generated from PIP_2 within 30 s after amputation in newt limbs [15] and that PKC rises to a peak by the accumulation blastema stage [16]. How these early signals are linked to the next phase of blastema formation, histolysis, and dedifferentiation, is unknown.

Histolysis and Dedifferentiation

Histolysis is the loss of tissue organization resulting from the enzymatic degradation of the extracellular matrix (ECM). Dedifferentiation is the reversal of a given state of differentiation to an earlier state via nuclear reprogramming and loss of specialized structure and function.

All of the tissues subjacent to the wound epidermis undergo intense histolysis (ECM degradation and tissue disorganization) for 1–2 mm, resulting in the liberation of fibroblasts, Schwann cells of the peripheral nerves, and skeletal cells [1]. Myofibers fragment at their cut ends and break up into mononucleate cells while releasing satellite cells (the stem cells that effect muscle regeneration). The liberated cells lose their phenotypic specializations and revert to mesenchymal-like cells with large nuclei and sparse cytoplasm that exhibit intense RNA and protein synthesis. Histolysis and dedifferentiation begin within 2–3 dpa in larval urodeles and within 4–5 days in adults.

Mechanisms of Histolysis

Degradation of tissue ECM is achieved by acid hydrolases and matrix metalloproteinases (MMPs) [17]. Acid hydrolases identified in regenerating urodele limbs include cathepsin D, acid phosphatase, β-glucuronidase, carboxyl ester hydrolases, and *N*-acetyl-glucosaminidase. Osteoclasts are abundant in the region of histolysis, where they degrade bone matrix via hydrochloric acid, acid hydrolases, and MMPs. Upregulated MMPs include MMP-2 and -9 (gelatinases), and MMP-3/10a and 10b (stromelysins) [18]. Macrophages are a major source of MMPs, particularly MMP-9 [19]. The basal layer of the wound epidermis is a source of MMP-3/10a and 10b in the newt limb, as well as of a novel MMP with low homology to other MMPs [20]. These MMPs are responsible for maintaining contact between the wound epidermis and the underlying tissues by preventing reassembly of a basement membrane. Chondrocytes are a source of MMP-2 and -9 in the newt limb, and these enzymes diffuse outward from the degrading skeletal elements [20]. The importance of MMPs to histolysis, and the importance of histolysis to the success of regeneration, are underscored by the failure of blastema formation in amputated newt limbs treated with an inhibitor of MMPs (GM6001) [21].

Histolysis continues to contribute blastema cells until the medium bud stage; then it ceases owing to the activity of tissue inhibitors of metalloproteinases (TIMPs) [18,22]. TIMP1 is upregulated during histolysis, when MMPs are at maximum levels, and exhibits spatial patterns of expression congruent with those of MMPs in the wound epidermis, proximal epidermis, and internal tissues undergoing disorganization.

Mechanisms of Dedifferentiation

Dedifferentiation is a complex and poorly understood process involving epigenetic reprogramming that suppresses the transcription of differentiation genes, while activating transcription of genes associated with stemness, reduction of cell stress, and remodeling internal structure. Inhibition of these transcriptional changes by actinomycin D does not affect histolysis, but it prevents or retards dedifferentiation, leading to regenerative failure or delay [23]. This suggests that at least part of the proteases involved in histolysis are not regulated at the transcriptional level, but that proteins effecting dedifferentiation are thus regulated. Dedifferentiated cells express a more limb bud–like ECM in which the basement membrane is absent, type I collagen synthesis and accumulation are reduced, and fibronectin, tenascin, and hyaluronate accumulate [24–26].

The molecular details of transcriptional regulation during dedifferentiation are largely unknown. Degradation of the ECM by proteases would break contacts between ECM molecules and integrin receptors, leading to changes in cell shape and reorganization of the actin cytoskeleton that might activate epigenetic reprogramming. Stemness genes upregulated during blastema formation are *msx1*, *nrad*, *rfrng*, and *notch* [17]. *Msx1* inhibits myogenesis [27] and its forced expression in mouse myotubes causes cellularization and reduced expression of muscle regulatory proteins [28]. Inhibition of *msx1* expression in cultured newt myofibers by anti-*msx* morpholinos prevents their cellularization [29]. Newt regeneration blastema extract stimulates mouse myonuclei to reenter the cell cycle, cellularize, and reduce their expression of muscle regulatory proteins [30]. *Nrad* expression is correlated with muscle

dedifferentiation [31], and *Notch* is a major mediator of stem cell self-renewal [32]. A micro-RNA gene regulatory circuit has been identified in regenerating axolotl limbs and fish fins [33].

Three of the six transcription factor genes (*klf4*, *sox2*, and *c-myc*) used to reprogram mammalian adult somatic cells to induced pluripotent stem cells (iPSCs) [34,35] are upregulated during blastema formation in regenerating newt limbs, and also during lens regeneration [36]. The Lin 28 protein, the product of a fourth transcription factor gene used to derive iPSCs [35], also is upregulated during blastema formation in regenerating axolotl limbs [13]. Thus, transcription factors that reprogram fibroblasts to iPSCs may also gave a role in nuclear reprogramming during limb regeneration. The further molecular characterization of transcription factors, micro-RNAs, and changes in epigenetic marks via chromatin-modifying enzymes will be crucial for understanding the mechanism of dedifferentiation in regenerating amphibian limbs.

The differential regulation of pathways that protect cells from stress and apoptosis also have a role in dedifferentiation. Proteomic analysis suggests that reduced metabolic activity, upregulation of pathways that accelerate protein folding or eliminate unfolded proteins (the unfolded protein response), and differential regulation of apoptotic pathways may largely prevent apoptosis [13], which is known to be minimal in regenerating limbs [37,38]. This idea is consistent with other studies on cultured chondrocytes, β cells, and Muller glia cells of the retina showing that cells dedifferentiate as part of a mechanism to combat apoptotic cell stress [13].

The molecular details of internal cellular remodeling are poorly understood. Dismantling of the phenotypic structure and function is most visible in myofibers, but the molecular details of the process are largely uninvestigated for any limb cell type. Two small molecules, one a trisubstituted purine called myoseverin and the other a disubstituted purine dubbed reversine, have been screened from combinatorial chemical libraries and have been found to cause cellularization of C2C12 mouse myofibers [39,40]. Myoseverin disrupts microtubules and upregulates genes for growth factors, immunomodulatory molecules, ECM remodeling proteases, and stress-response genes, which is consistent with the activation of pathways involved in wound healing and regeneration, but it does not activate the whole program of myogenic dedifferentiation in newt limbs [41]. Reversine treatment of C2C12 myotubes resulted in mononucleate cells that behaved like mesenchymal stem cells, i.e., they were able to differentiate in vitro into osteoblasts and adipocytes as well as muscle cells [42]. Myoseverin and reversine might be useful in analyzing the events of structural remodeling, and may have natural counterparts that can be isolated.

Differential Tissue Contributions to the Blastema

Individual tissues of the limb make differential contributions to the blastema. In the axolotl limb, dermal fibroblasts represent 19%, and chondrocytes 6%, of the cells present at the amputation surface [43]. Dermal fibroblasts contribute nearly half of the blastema cells; fibroblasts of the periosteum and myofiber/nerve sheath interstitial connective tissue, Schwann cells, and myogenic tissue contribute the rest. Experiments transplanting green fluorescent protein (GFP) cartilage into a limb wound induced the formation of a supernumerary limb, which suggests that chondrocytes make no contribution to the blastema [44]. Transplants of other GFP-labeled tissues have shown that the redifferentiation of blastema cells is largely lineage-specific, i.e., they are constrained to reproduce their parent cell types. The exception is connective tissue fibroblasts, which after dedifferentiation are able to transdifferentiate at high frequency into cartilage [45–47]. Lineage-restricted redifferentiation is also reflected in stably maintained histone methylation patterns of parent cells [48]. Thus, although transcription factors that reprogram fibroblasts to iPSCs have a role in nuclear reprogramming during limb regeneration, other factors clearly ensure that dedifferentiated cells reverse their transcription programs only far enough to attain a state that can respond to proliferation and patterning signals while maintaining their phenotypic memory of origin.

There are developmental and species differences in muscle contributions to the blastema. Muscle regenerates in larval and adult axolotl and larval newt limbs by $Pax7^{+}$ satellite cells, whereas in adult newts, muscle regenerates via fragmentation of the ends of cut myofibers and dedifferentiation of the resulting mononucleate cells [49,50]. Whether the switch from muscle regeneration via satellite cells in newt larvae to dedifferentiation of myofiber fragments in adults is all-or-none or gradual is unknown.

Cell Cycling During Blastema Formation

Tritiated thymidine (^{3}H-T) labeling studies have shown that cells of amputated urodele limbs initiate cell cycle entry coincident with their liberation by histolysis. The pulse-labeling index reaches 10–30% during formation of the accumulation blastema [51,52]. However, the mitotic index is low, between 0.1% and 0.7% (average of about 0.4%, or 4 in

1000 cells) in both *Ambystoma* larvae [53] and adult newt [54]. The low mitotic index during establishment of the accumulation blastema suggests that it forms primarily by accumulating dedifferentiated cells rather than their mitosis.

The fact that cells readily enter the cell cycle during formation of the accumulation blastema but divide only infrequently suggests that a large proportion of dedifferentiating cells arrest in G_2 (M51). Further indirect evidence for G_2 arrest is the strong upregulation of the ecotropic viral integration factor 5 (Evi5) throughout blastema formation in regenerating axolotl limbs [13]. Evi5 is a centrosomal protein that accumulates in the nucleus during early G_1 in mammalian cells and prevents them from prematurely entering mitosis by stabilizing Emi1, a protein that inhibits cyclin A degradation by the anaphase-promoting complex/cyclosome [55]. At G_2, Emi1 and Evi5 are phosphorylated by Polo-like kinase 1 and targeted for ubiquitin-driven degradation, allowing the cell to enter mitosis. Thus, high levels of Evi5 during blastema formation may restrain cells from entering mitosis until they are fully dedifferentiated and present in enough numbers to form an accumulation blastema [13]. To test this hypothesis, it will first be necessary to determine the spatiotemporal expression pattern of Emi1 and Evi5. The hypothesis predicts that these proteins would be expressed at high levels in both wound epidermis and blastema mesenchyme, and that expression would decrease as the cells transited a normal cell cycle.

The signals that drive liberated cells to enter the cell cycle have been studied in detail in myofibers of the regenerating newt limb. Entry into the cell cycle of muscle-derived blastema cells appears to be initiated by the muscle LIM protein (MLP), a member of the MARCKS family that has a role in muscle differentiation [56]; whether MLP initiates cell cycle entry of blastema cells derived from other limb tissues is unknown. Progression through G1 and S in cultured newt and mouse myoblasts and newt myofibers is promoted by a thrombin-activated factor present in the serum of all vertebrates tested thus far, including mammals, that deactivates the Rb protein [57]. Mouse myofibers do not respond to this factor. Newt blastema extract promotes DNA synthesis in both newt and mouse myofibers [30], which suggests that mouse myofibers lack an essential signal pathway ingredient that is supplied by newt blastema extract but not by serum. Although the thrombin-activated protein is both necessary and sufficient to stimulate the entry of myonuclei into the cell cycle, it is not sufficient to drive them through mitosis, and myonuclei arrest in G_2. Cell cycle reentry is independent of myofiber cellularization, because cell cycle−inhibited myofibers implanted into newt limb blastemas break up into mononucleate cells [58]. However, mitosis appears to require mononucleate cell status. The mechanism of myofiber fragmentation into single cells is not known, nor is it known whether the thrombin-activated protein is also necessary to drive cells such as dedifferentiating fibroblasts and Schwann cells into the cell cycle or whether this is a feature unique to myofibers. Biochemical evidence suggests that the thrombin-activated factor may be a potent growth factor required in very small amounts [57].

Macrophages Have an Important Role in Blastema Formation

Macrophages of the innate immune system are important mediators of wound repair in mammals via their bactericidal and phagocytic activities and their secretion of growth factors and cytokines that modulate inflammation and initiate structural repair by fibroblasts [17]. Studies emphasize the importance of the immune system, particularly macrophages, for the events of blastema formation during urodele limb regeneration [59−61].

Proinflammatory and antiinflammatory cytokines are upregulated during blastema formation in regenerating limbs of adult axolotls, coincident with a significant enrichment of macrophages, which produce both types of cytokines as well as MMPs. Macrophage depletion by liposome-encapsulated clodronate during blastema formation results in regenerative failure and scarring of the limb stump. The epidermis closes the wound but does not develop an AEC, and dermal scar tissue is interposed between the wound epidermis and underlying tissues [19]. By contrast, depletion after a blastema enters the growth phase only delays regeneration. These results suggest a central role for macrophages in resolving inflammation and the degradation of ECM. Macrophages have also been shown to have a role in removing senescent cells during urodele limb regeneration. In mammals, senescent cells accumulate in tissues with age. Few cells undergoing apoptosis are detected in regenerating urodele limbs [37]. Cell senescence is induced during blastema formation in regenerating urodele limbs, but senescent cells are cleared by macrophages and do not accumulate [62].

Blastema Cell Migration and Accumulation

The AEC appears to direct the migration of blastema cells to aggregate beneath it [1]. This was shown by experiments in which shifting the position of the AEC laterally caused a corresponding shift in blastema cell accumulation, and transplantation of an additional AEC to the base of the blastema resulted in supernumerary blastema

formation. Nerve guidance of blastema cells to form eccentric blastemas appeared to be ruled out, because similar experiments on aneurogenic limbs resulted in eccentric blastema formation.

The redirected accumulation of blastema cells in these experiments results from the migration of the cells on adhesive substrates produced by the eccentric AEC. Transforming growth factor (TGF)-β1 is strongly upregulated during blastema formation in amputated axolotl limbs [63]. A target gene of TGF-β1 is fibronectin, a substrate molecule for cell migration that is highly expressed by basal cells of the wound epidermis during blastema formation [13,64]. Inhibition of TGF-β1 expression by the inhibitor of SMAD phosphorylation, SB-431542, reduces fibronectin expression and results in failure of blastema formation [63]; this suggests that fibronectin provided by the AEC provides directional guidance for blastema cells.

BLASTEMA GROWTH

There are two synergistic requirements for cells of the accumulation blastema to break G_2 arrest, enter mitosis, and proliferate. The first is the production of mitogens via an interaction of the AEC with nerve axons. The second is that cells of the accumulation blastema derived from opposite positions on the limb circumference must migrate toward one another and interact. Unless these two conditions, along with vascularization, are met, the accumulation blastema will not persist and will regress.

The Apical Epidermal Cap–Nerve Interaction

Neither denervation at the time of amputation nor deprivation of wound epidermis prevents histolysis and formation of blastema cells, but the blastema cells do not persist [65]. The ^{3}H-T labeling index is the same as that of controls during formation of the accumulation blastema in both epidermis-free and denervated limbs, which suggests that neither the nerve nor the wound epidermis is required for DNA synthesis during this time [53,66–68].

The AEC of the accumulation blastema is invaded by sprouting sensory axons as it forms, whereas other sensory axons and motor axons make intimate contact with mesenchyme cells of the blastema [69]. Coincident with innervation of the AEC, the labeling and mitotic indices of the accumulation blastema rise as much as 10-fold (Fig. 3.2) and the blastema enters the phase of growth and patterning [51,52]. These increases do not take place in denervated or wound epidermis–deprived limbs. ^{3}H-T pulse labeling studies indicate that the final cycling fraction of blastema cells is between 92% and 96% in larvae and over 90% in adults [70,71]. These results are consistent with the idea that blastema formation results from cell migration and aggregation, not mitosis.

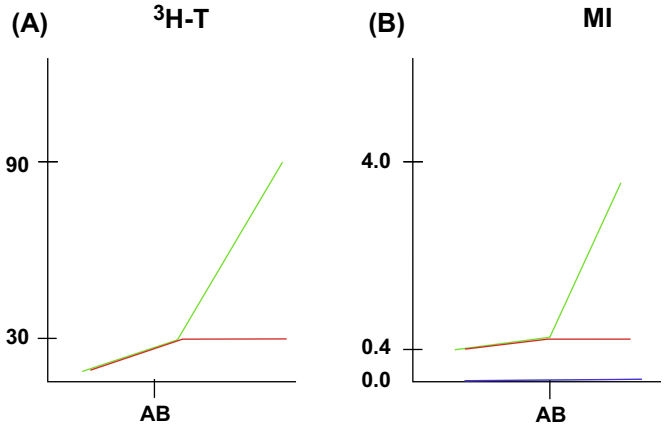

FIGURE 3.2 Diagram of changes in tritiated thymidine (^{3}H-T) labeling and mitotic index (MI) during blastema formation and growth, expressed as percentages of the total cell number on the ordinate. AB on the abscissa represents the accumulation blastema stage. The growth phase is to the right of the AB. (A) Before the accumulation blastema stage, the ^{3}H-T labeling index is the same in control (*green line*) and in epidermis-free and denervated limbs (both represented by the *red line*). These indices in deprived limbs fail to rise in concert with the controls during blastema growth and an accumulation blastema does not form. (B) Before the accumulation blastema stage, the basal mitotic index of controls (*green line*) and epidermis-free limbs (*red line*) are nearly identical, but the MI does not increase with the controls during blastema growth. In contrast, the MI in denervated limbs (*blue line*) does not achieve the basal level and remains near zero. An accumulation blastema does not form in either denervated or epidermis-free limbs.

When the growing blastema achieves a critical mass of cells, it becomes independent of the nerve for its differentiation and morphogenesis, but its individual cells remain nerve-dependent for mitosis [72,73]. Thus, a blastema denervated at the medium bud stage will form a morphologically normal but miniature regenerate owing to the lack of further mitosis. An established blastema mesenchyme stripped of its epidermis by chelation and denervated by implanting it in a dorsal fin tunnel such that its distal end becomes recovered by fin epidermis also forms a morphologically normal but miniature regenerate. Consistent with this result, the ^{3}H-thymidine labeling and mitotic indices of epidermis-free newt limb blastemas cultured in the presence of dorsal root ganglia are reduced three- to fourfold [74]. If the mesenchyme is implanted completely into the fin tunnel, however, a miniature regenerate forms that is distally truncated [75]. This result suggests that the AEC has a role in proximodistal (PD) patterning in addition to proliferation.

As in other vertebrate embryos, the amphibian limb bud requires signals from the AEC for outgrowth that are generated by a reciprocal interaction between mesenchyme and AEC. It becomes nerve-dependent for regeneration only after it has differentiated and become innervated [76]. Urodele limb buds rendered aneurogenic are AEC-dependent for growth but do not become nerve-dependent for regeneration [77,78], although this dependence can be instituted by allowing the limbs to become reinnervated [79]. These facts suggest a model for blastema growth in which the AEC supplies mitogens to subjacent blastema cells but requires factors supplied by the nerve to perform this function (Fig. 3.3).

If this model outlined is correct, putative AEC and nerve factors should meet several criteria, in addition, to being expressed by the AEC and nerves. For AEC candidates, these are expression of their receptor in the blastema mesenchyme, loss of AEC expression by denervation, and the ability to support regeneration of denervated or AEC-deprived limbs to digit stages. Neural factors should be transported from nerve cell bodies along limb nerve axons to the AEC where they bind to their receptor, denervation should prevent blastema cell mitosis by abolishing AEC factors, and they should support regeneration to digit stages in denervated limbs.

Many factors expressed by the AEC stimulate blastema cell proliferation in vitro and in vivo. Fibroblast growth factor (Fgf)1, Fgf2, and the anterior gradient (AG) protein are expressed by the AEC in vivo [80,81], and mesenchymal cells express receptors for Fgfs and AG [81,82]. Fgf1 elevated the mitotic index of cultured blastema cells [83], and Fgf2 elevated the mitotic index of blastema cells in amputated limbs covered by full-thickness skin [84]. However, only two factors expressed by the AEC have been shown to be downregulated by denervation and to substitute for the nerve in supporting regeneration to digit stages. These are Fgf2 [85] and AG [81]. AG is involved in head development of the *Xenopus* embryo and has been the more thoroughly investigated of the two. It is strongly

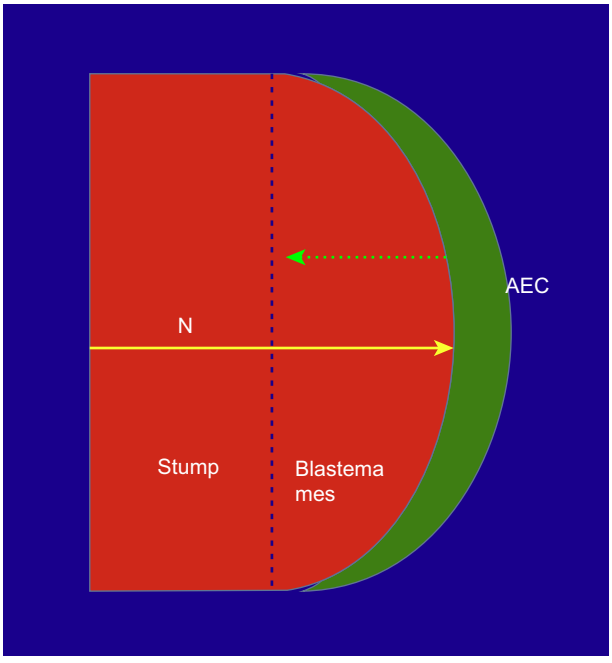

FIGURE 3.3 Model of apical epidermal cap (AEC)—neural (N) interaction for mitotic growth of the blastema. The function of the AEC is to secrete mitogens into the subjacent blastema mesenchyme (mes) (*green dashed line*); this function depends on factors made by dorsal root ganglion neurons (*solid yellow line*). *Dashed blue line* indicates level of amputation.

expressed in Schwann cells insulating the axons of regenerating newt limbs at 5 and 8 dpa, when initial dedifferentiation is under way. By 10 dpa, AG expression shifts to the gland cells of the AEC, coincident with formation of the accumulation blastema. Denervation abolishes AG expression, indicating that it is induced by axons.

The AG gene supports regeneration to digit stages when electroporated into denervated newt limbs at 5 dpa. The receptor for AG is the blastema cell surface protein Prod1, a member of the Ly6 family of three-finger proteins anchored to the cell surface by a glycosylphosphatidylinositol linkage [86]. Conditioned medium of Cos7 cells transfected with the AG gene stimulates 5-bromo-2′-deoxyuridine incorporation into cultured blastema cells; this incorporation is blocked by antibodies to Prod1, which suggests that AG acts directly on blastema cells through Prod1 to stimulate proliferation [81]. The dependence on nerves for mitosis throughout blastema growth implies that this action is continuous, an implication that could be confirmed or refuted by examining expression patterns of AG in control and denervated limbs at successively later stages of blastema redevelopment. As would be predicted by the nerve−AEC model, AG is expressed in developing urodele limb buds and regenerating aneurogenic limbs [87]. Fgf2 expression in the AEC of aneurogenic limbs has not been examined.

Factors expressed by DRG neurons that promote blastema cell proliferation in vitro include transferrin, substance P, Fgf2, and glial growth factor 2 (Ggf-2) [17,88]. Ggf-2 was reported to rescue regeneration to digit stages in denervated axolotl limbs when injected intraperitoneally during blastema formation [89], but this has not been investigated further. New nerve factor candidates are combinations of Fgf8 and bone morphogenetic protein. Both are expressed in DRG neurons and are detectable in peripheral limb nerve axons [90]. Furthermore, they can substitute for the nerve in inducing a supernumerary limb [91].

Interaction of Cells From Opposite Sides of the Limb Circumference

Blastema cells also fail to persist unless they are derived from opposite positions on the limb circumference and interact. This was shown by experiments in which the normally asymmetrical (anteroposterior [AP] or dorsoventral [DV]) skin of the newt limb was made symmetrical by rotating a longitudinal strip of skin 90 degrees, grafting it around the circumference of an irradiated limb, and then amputating through the strip [92]. This led to failure of blastema cell mitosis, which indicates that mitosis requires an interaction between blastema cells with opposite positional identities. Normal regeneration ensued, however, when this requirement was met by grafting short longitudinal skin strips from two or more opposite points of the circumference.

Lheureux [93] devised an experimental model to show the synergistic effect on mitosis of nerve-induced mitogen expression by the AEC with the interaction between cells of opposite positional identities. He made a wound on one side of an adult newt limb, deviated a nerve to the wound site, and juxtaposed a graft of skin from the opposite side of the limb to the skin of the wound. By themselves, nerve deviation or juxtaposing opposite positional identities resulted in the formation of blastema cells that failed to persist. Together, however, they stimulated the formation of a supernumerary blastema that grew and regenerated a complete limb. The Lheureux model was later used by another group to evoke supernumerary blastema formation in axolotl limbs under the name “Accessory Limb Model” [94]. Supernumerary limbs can be induced in the same way by reversing the AP or DV axis of the blastema with respect to the limb stump.

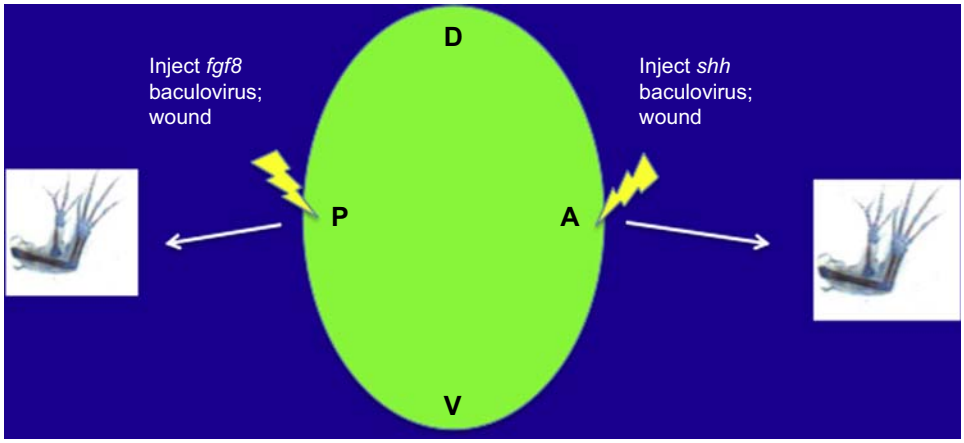

FIGURE 3.4 Cross-section of axolotl stylopodium and result of injecting baculovirus constructs containing *Fgf8* under posterior skin and *Shh* under anterior skin, followed by wounding and nerve deviation. *Fgf8* and *Shh* substitute for anterior and posterior skin, respectively, in evoking supernumerary limb formation. *A*, anterior; *D*, dorsal; *P*, posterior; *V*, ventral.

The Lheureux system has been used to investigate several aspects of limb regeneration. One of these has been to define the signals passing between cells of opposite positional identity. Nacu et al. [95] injected the sonic hedgehog (*shh*) gene under anterior skin of the stylopodium and the *fgf8* gene under posterior skin using a baculoviral vector system (Fig. 3.4). Shh and Fgf8 have been implicated in AP patterning of the limb bud and regeneration blastema [96,97]. A wound was then created in the skin and a nerve deviated to the site. The gene transfections substituted for cells of opposite positional identity, which indicated that Shh and Fgf8 signal opposite cells to drive some aspect of mitosis. The AEC does not persist in the absence of polar juxtaposition, which suggests that proliferating blastema cells may also be essential for AEC maintenance.

Positional identity in the PD axis of the limb is associated with a proximal (low) to distal (high) gradient of cell adhesion [98–100]. Prod-1 is expressed in an opposite gradient; antibodies to Prod1, or its removal from the blastema cell surface by phosphatidylinositol-specific phospholipase C inhibit the recognition of adhesive differentials between distal and proximal blastemas [86]. These results suggest that Prod-1 has a role in integrating mitosis with patterning through its AG ligand [101]. The PD segments of the regenerating limb appear to be specified in a proximal to distal direction [102], but how the positional identities reflected in cell surface adhesion are generated is not clear. There is evidence that PD patterning of the chick limb bud switches from an extrinsic signaling mechanism to an intrinsic timing mechanism that is reflected in intrinsic changes in cell affinities in the distal limb bud mesenchyme [103]. Whether blastema patterning uses a similar mechanism is unknown but would be worth investigating.

List of Acronyms and Abbreviations

AEC Apical epidermal cap
AG Anterior gradient
AP Anteroposterior
DRG Dorsal root ganglion
DV Dorsoventral
ECM Extracellular matrix
GFP Green fluorescent protein
IP_3 Inositol triphosphate
MMP Matrix metalloproteinase
PD Proximodistal
PKC Protein kinase C
Shh Sonic hedgehog
TIMP Tissue inhibitor of metalloproteases

Glossary

Aneurogenic limb A limb that develops without innervation.
Blastema The collection of undifferentiated cells that forms after amputation of a limb.
Dedifferentiation The process by which differentiated cells revert to progenitor cells.
Fibroblastema A blastema composed of fibroblastic cells instead of dedifferentiated cells.
Growth factors Proteins that promote cell proliferation and differentiation.
Histolysis The breakdown of extracellular matrix to release cells.
Satellite cell The progenitor cells that regenerate injured muscle.
SMAD Proteins phosphorylated by activation of TGF-b/BMP receptors that participate in activating or repressing transcription.
Stem cell Undifferentiated cells that maintain or repair tissue structure.
Transdifferentiation The differentiation of one cell type into another cell type.

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CHAPTER

4

The Molecular Circuitry Underlying Pluripotency in Embryonic and Induced Pluripotent Stem Cells

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INTRODUCTION

Multiple criteria are employed to characterize cellular pluripotent potential, including (1) the expression of molecular markers, in particular, transcription factors known to regulate embryonic stem cell (ESC) potency and self-renewal; (2) the absence of molecular and morphological markers defining specific lineages that are typically referred to as "differentiation-associated genes"; and (3) the ability to form all three embryonic germ layers including ectoderm, endoderm, and mesoderm upon induction of differentiation in vitro or in vivo. Upon injection into immunocompromised mice, ESCs and induced pluripotent stem (iPS) cells will rapidly and reproducibly form teratomas containing differentiated cells from the three germ layers. Ultimately, the most rigorous pluripotency assay, but one not currently amenable to studying human cells, for ethical reasons, is that upon implantation of ESCs into blastocysts there is a subsequent contribution of these cells to all tissue types of the adult chimeric animal. Although some stem-like cells have been isolated from many organisms, including zebrafish, dogs, chickens, mice, rats, and humans, to date only stem cells from a few species have demonstrated this ultimate and most stringent capability associated with pluripotency by which they can be defined concretely as ESCs.

In this review, we describe the mechanistic details of the molecular circuitry that regulates the maintenance of the pluripotent state at the level of signal transduction, chromatin dynamics, and transcription factor control. We also discuss the reprogramming of somatic cells into iPS cells that possess essentially all ESC-like properties, and how the processes that govern their production and maintenance relate to ESC maintenance. Analyses of the rewiring of the molecular circuitry involved in going from a somatic to pluripotent state have reinforced the identities and roles of core pluripotency mechanisms.

GROUND STATE AND PRIMED EMBRYONIC STEM CELLS HAVE UNIQUE SIGNALING NETWORKS UNDERLYING PLURIPOTENCY

The initial protocols employed to isolate and maintain murine ESCs involved plating inner-cell mass cells onto a feeder cell layer of embryonic fibroblasts in a medium containing serum proteins [1,2]. The complex mixture of exogenous factors released by fibroblasts into the medium maintains ESCs in their pluripotent state and allows for the undifferentiated self-renewal and proliferation of these cells. Despite the complex composition of fibroblast-conditioned medium containing serum components, several key growth factor signaling pathways essential for subsequent pluripotency have been identified and extensively characterized, including leukemia inhibitory factor (LIF)/signal transducer and activator of transcription 3 (Stat3), and bone morphogenic protein (BMP) signaling. Upon removal of the feeder cells, or medium conditioned by the feeder cells, ESCs spontaneously differentiate into all three germ layers of the developing organism. More recent protocols have been developed and used

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alternative media options, including 2i and 3i media that allow for feeder-independent growth of mouse ESCs (mESCs) [3]. Development of these media was based on the idea that inhibition of intrinsic differentiation signals could maintain pluripotency, similar to the application of exogenous factors (from fibroblast feeders and serum). For instance, 2i medium uses an inhibitor of mitogen activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) (MEK) combined with an inhibitor of glycogen synthase kinase 3β (Gsk3β) and does not require LIF for stem cell maintenance [4].

Human ESCs (hESCs) can be grown on feeder cells in media conditioned by fibroblasts or in chemically defined media [5]. Interestingly, studies with these human cells revealed striking differences in signaling pathway requirements for stem cell maintenance compared with mESCs, which led to the hypothesis that despite the high levels of conservation in development across widely divergent species, pluripotent stem cell maintenance is at least partially a unique process in different organisms. This line of thinking has been challenged by more recent findings that distinguish two main states of ESCs: naive and primed. It was found that under commonly used derivation and culture conditions, mESCs are maintained in the naive state whereas hESCs acquire and maintain the primed state [6]. Primed mESCs, which are derived from the mouse embryo after implantation and are typically called epiblast stem cells (EpiSCs), share many of the same signaling pathway requirements as do hESCs grown under standard conditions [7]. Several articles reported the development of media and conditions that allow for culturing of naive hESC [8,9] and found in this context that the pathway requirements are much more similar to those required by naive mESC, including the dependency on LIF. These findings suggest LIF signaling is a key aspect of naive pluripotent stem cell self-renewal across species. Thus, apparent differences in human and murine ESCs may be largely a function of the developmental state related to how the cells are cultured, which signaling pathways are modulated in the process, and thus which cell fate–related transcription factors are activated or repressed downstream.

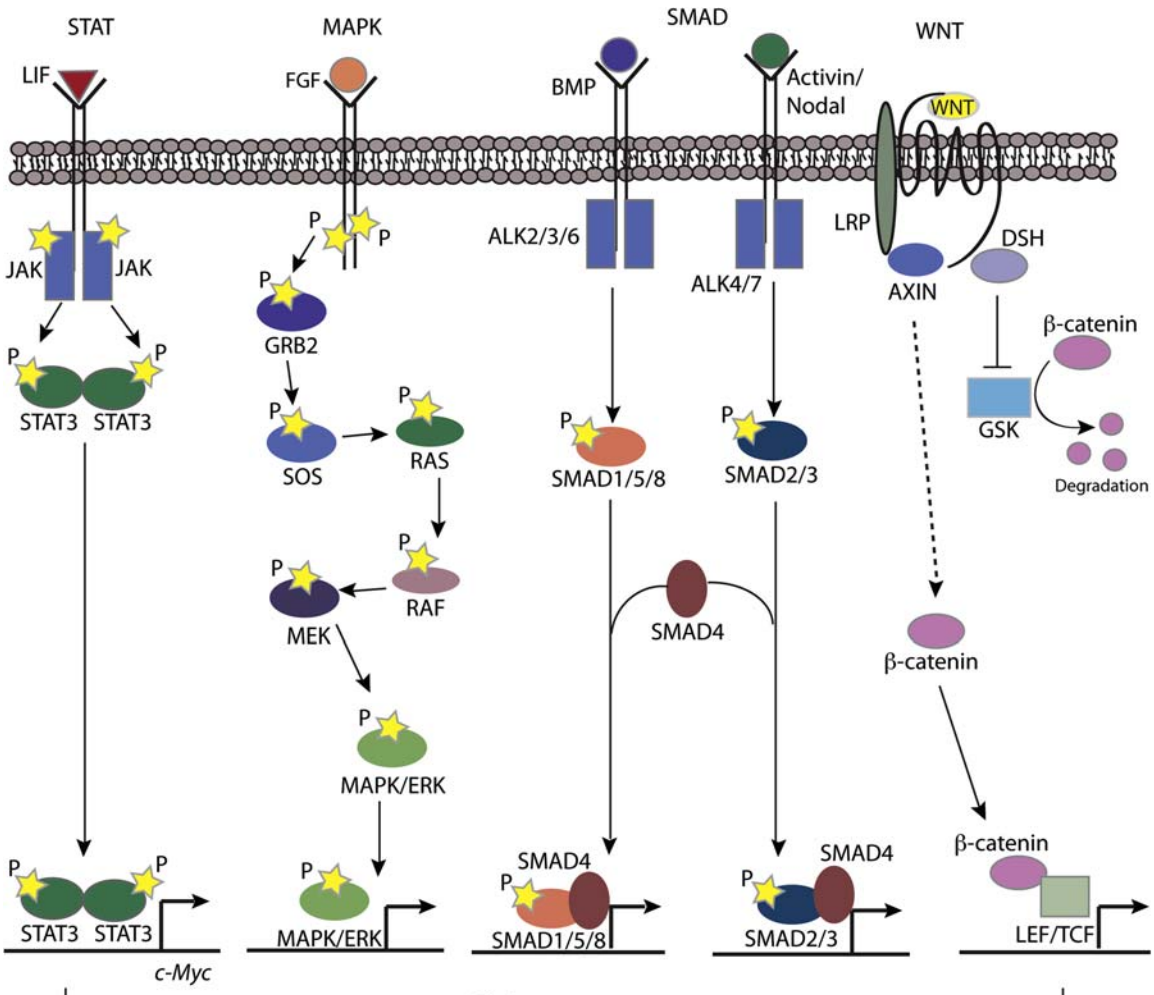
INDUCED PLURIPOTENT STEM CELLS

An additional type of pluripotent stem cells, iPS cells, can be derived from human or mouse somatic cells through related protocols mainly based on exogenous expression of a select group of pluripotency-related transcription factors. In 2006, Yamanaka and colleagues reported that by introducing four transcription factors necessary for ESC self-renewal including *Oct4*, *Sox2*, *Klf4*, and *c-Myc* into the genome of mouse fibroblasts, some cells underwent complete reprogramming to a state of pluripotency [10]. Further work including studies by Yu et al. found that *Klf4* and *c-Myc* can be substituted by *Nanog* and another transcription factor *Lin28* [11], or that the entire reprogramming process in humans and mice can be accomplished through the expression of a single cluster of microRNAs (miRNAs), mir302/367 [12]. These results indicate that multiple combinations of transcription factors or other regulators can reprogram cells to the same primitive developmental state [11]. A year later, Yamanaka and other teams reported successful human somatic cell reprogramming to make iPS cells [13]. These human iPS cells possess all of the hallmarks of hESCs in their functional abilities to differentiate into all cell types of an organism [10]. Importantly, these distinct pluripotent cells also require the same signaling pathways to maintain their undifferentiated state and respond appropriately to growth factors and cytokines eliciting specific lineages [14].

Reprogramming methods have been developed including the use of nongenetic methods that do not leave a genomic fingerprint via episomal vectors, nonintegrating virus, messenger RNAs (mRNAs), proteins, and even in an entirely chemical cocktail [15–17]. Although the reprogramming protocols are well-established, large fluctuations remain in efficiency and the probability that any given cell will become an iPS cell. Even among a clonally selected somatic cell population infused with the same copy number of reprogramming factors, heterogeneity abounds, with a minority of cells reprogramming within a few weeks while others require longer periods [18]. Part of the answer to the heterogeneity may reside in the concept of nongenetic heterogeneity and random fluctuations in protein expression levels among a clonal population of cells; heterogeneity in any number of transcription factors, signaling proteins, or epigenetic marks may place a cell in a state either relatively amenable or resistant to proper manipulation by the reprogramming factors.

LEUKEMIA INHIBITORY FACTOR AND BONE MORPHOGENIC PROTEIN SIGNALING PATHWAYS REGULATE MOUSE EMBRYONIC STEM CELL SELF-RENEWAL

mESCs derived from permissive strains can maintain their undifferentiated state with a combination of signaling from LIF and BMPs (Bmp4) [19,20]. LIF receptor activation leads to receptor dimerization with gp130 subunits and subsequent tyrosine phosphorylation and nuclear localization of the transcriptional activator Stat3 (Fig. 4.1) [21].



	LIF/STAT	FGF/MAPK	BMP	TGF-β	WNT
naive mESC	+	-	+	-	+
primed mESC (EpiSC)	-	+	-	+	-
naive hESC	+	+	-	+	+
primed hESC	-	+	-	+	+

FIGURE 4.1 **Signaling circuitry regulating mouse and human embryonic stem cell (ESC) pluripotency.** The Wnt pathway is a highly conserved regulator of pluripotency and is active in both mouse and human ESCs. Different signaling pathways are required to maintain pluripotency, depending on the state of the stem cells and species. Naive mESCs require leukemia inhibitory factor (LIF) and bone morphogenic protein (BMP) signals to maintain self-renewal, whereas primed mESCs require transforming growth factor β (TGFβ) and fibroblast growth factor (FGF) signaling. Naive human ESCs (hESCs) have been derived using various media formulations, but in general they require LIF, FGF, TGFβ, and WNT signaling. Primed hESCs depend on the activity of TGFβ and FGF signals. These pathways ultimately function at multiple levels to maintain the pluripotent state by inhibiting differentiation and feeding into the core transcriptional regulatory circuitry of ESCs. *ALK*, anaplastic lymphoma kinase; *EpiSC*, epiblast stem cells; *ERK*, extracellular signal—regulated kinase; *GSK*, glycogen synthase kinase; *MAPK*, mitogen activated protein kinase; *MEK*, MAPK/ERK; *mESC*, mouse ESC; *STAT*, signal transducer and activator of transcription; *TCF*, transcription factor

Whereas LIF also activates the phosphatidylinositol-3-kinase/protein kinase B and MAPK signaling pathways in mESCs, only Stat3 activation is required for pluripotency [22]. One of the key downstream targets of LIF/Stat3 is the transcription factor *Myc* (Fig. 4.1) [23]; high levels of *Myc* expression even in the absence of LIF signaling allows for self-renewal of mESCs [23], which suggests that the main downstream target of the LIF/Stat3 signaling pathway in mESC is *c-Myc*.

BMPs are transforming growth factor β (TGFβ) superfamily members that bind to type I TGFβ receptors anaplastic lymphoma kinase (ALK)1, ALK2, ALK3, or ALK6 (Fig. 4.1). Upon ligand binding, type I receptors form heterodimers with type II receptors, which recruit and phosphorylate receptor activated SMADs 1, 5, and 8 (R-SMADs). Serine/threonine phosphorylation of R-SMADs allows association and complex formation with SMAD4, which can subsequently enter the nucleus and initiate transcription (Fig. 4.1) [24]. Among the targets of BMP signaling is Inhibitor of Differentiation 1 (*Id1*), a key factor that can maintain ESC self-renewal when overexpressed even in the absence of BMP signaling [19]. In addition, chromatin immunoprecipitation (ChIP)-sequencing (Seq) studies revealed that Smad1 colocalizes with octamer binding protein 4 (Oct4), SRY-box 2 (Sox2), Nanog, and Stat3 at a number of pluripotency gene targets [25].

Whereas ERK has generally been considered to promote differentiation of mESCs (it promotes Kruppel-like factor 4 (Klf4) ubiquitination and degradation [26] and reduces Nanog's activating ability [27], and inhibitors of upstream factor MEK are used to maintain mESC pluripotency), it has also been shown to have an important role in maintaining genomic stability, promoting proliferation, and suppressing apoptosis in mESCs [28]. This suggests a more complex role for this factor in stem cell dynamics.

TRANSFORMING GROWTH FACTOR β AND FIBROBLAST GROWTH FACTOR SIGNALING PATHWAYS REGULATE HUMAN EMBRYONIC STEM CELL SELF-RENEWAL

As discussed earlier, hESCs cultured on fibroblast feeder cells with serum exist in a more primed state, with similarities to mouse EpiSCs. LIF/STAT3 signaling does not stimulate self-renewal in this state; rather, TGFβ/activin and fibroblast growth factor (FGF) signaling are required to maintain hESC pluripotency [14,29]. In contrast to naive-state mESCs, BMP signaling actually promotes hESC differentiation [30,31]. TGFβ and activins account for the second branch of the TGFβ superfamily of ligands, and binding to receptors ALK4, ALK5, and ALK7 triggers serine/threonine phosphorylation of the C-terminal region of SMAD2 and SMAD3, which also dimerize with SMAD4 to allow nuclear entry and transcription (Fig. 4.1) [24]. FGFs function through tyrosine receptor dimerization upon ligand binding and subsequent activation of phosphorylation events in the MAPK cascade (Fig. 4.1) [32]. FGF signaling can further phosphorylate both BMP and TGFβ-mediated R-SMADs at the "linker" domain of the proteins. This phosphorylation has been associated with signal termination, because linker phosphorylation allows recognition of SMAD proteins by the ubiquitin ligase SMURF1 [33,34]. Polyubiquitination of the SMAD proteins by SMURF1 leads to subsequent degradation of the SMAD proteins and termination of the signal. Hence, an intricate balance of antagonistic signaling inputs may exist in the maintenance of hESC pluripotency. Among their definitive roles in proliferation and survival, FGF signals may also act to inhibit differentiation promoting BMP signals in hESCs by promoting degradation of any active SMAD 1/5/8 proteins [33]. Alternatively, FGF signals may also fine-tune the amount of active TGFβ-mediated SMAD 2/3 proteins to produce the proper threshold of activity necessary to maintain pluripotency, because an excess of TGFβ/activin signaling can lead to definitive endoderm differentiation of ESCs [35].

Studies demonstrating the necessity of these pathways to maintain self-renewal have followed two strategies. First, small molecule inhibition of TGFβ/activin receptors results in the rapid differentiation of hESCs even in fibroblast-conditioned medium, which illustrates the necessity for TGFβ signals to maintain pluripotency [29]. Second, defined medium with select growth factors and cytokines has been developed to substitute fibroblast-conditioned medium, which contains a diverse milieu of undefined components. These studies revealed that TGFβ or activin plus FGF-2 at defined concentrations is a necessary component for self-renewal, and removal of either of these factors results in differentiation of ESCs [14,36]. For both mouse and human iPS cells, the same media and growth factor pathway activities are required for culture and maintenance of their pluripotency as for the respective comparable types of ESCs, which further supports the important nature of these pathways in pluripotency.

WNT SIGNALING CONTRIBUTES TO MAINTENANCE OF PLURIPOTENCY IN MOUSE EMBRYONIC STEM CELLS AND TO THE NAIVE HUMAN EMBRYONIC STEM CELL STATE

Although activation of LIF and BMP signaling are sufficient to maintain mESC self-renewal, the highly conserved Wnt pathway is also an important modulator of these signaling pathways; it can contribute to the maintenance of mESC pluripotency [37–39]. In the presence of Wnt ligand, a receptor complex forms between receptors Frizzled

and Lrp5/6. This complex recruits and sequesters Axin and Gsk3β, releasing their inhibitory interaction with β-catenin, which subsequently can accumulate in the nucleus, where it serves as a coactivator to activate Wnt-responsive genes (Fig. 4.1) [40]. β-Catenin also interacts with chromatin remodeling factor Brg1 to remodel chromatin during gene activation in response to Wnt signaling [41]. β-Catenin is important for maintaining stem cell identity and inhibiting neuronal differentiation, because mESCs that lack this factor show decreased expression levels of stem cell genes *Dppa4*, *Dppa5*, and *Rex1*, and increased expression of *Oct6*, a marker of neuronal differentiation [42].

Functional studies demonstrating the importance of Wnt signaling have employed small molecule inhibitors of Gsk3β, which destabilizes β-catenin. Inhibition of Gsk3β results in increased Wnt activity, and cells cultured in the presence of Gsk3β inhibitors have increased propensity to maintain their pluripotent state even under differentiation conditions [19,37]. Furthermore, the role of Wnt ligands in supporting stemness has been demonstrated in experiments that show that Wnts secreted by feeder cells or Wnt-conditioned media maintain pluripotency in mESCs [38,39].

Extensive cross-talk has been documented between the LIF and Wnt signaling pathways; when LIF is removed from culture media, mESCs reduce levels of nuclear β-catenin [43]. In addition, Wnt signaling molecules Wnt3a, Wnt5a, and Wnt6 can upregulate Stat3 signaling (downstream of LIF), and upregulation of Wnt signaling can compensate for low levels or the complete absence of LIF; LIF presence can also compensate for the loss of β-catenin [38]. Because β-catenin knockout mESCs have been shown to be able to self-renew in the presence of LIF, it appears not to be absolutely essential to the maintenance of pluripotency; rather, it acts in concert with other factors to enhance stem cell self-renewal. This idea is supported by the finding that Wnt signaling is also important in reprogramming differentiated cells into iPS cells, because Wnt3a can improve reprogramming efficiency [44].

Transcription factor 3 (Tcf3) is a transcription factor and binding partner for β-catenin. It has been shown to bind to many pluripotency genes and act as a repressor. It is also commonly found in complex with Oct4, Sox2, and Nanog, where it is thought to have a role in tempering the level of activation by these factors at pluripotency genes [45,46]. Upon stimulation of Wnt signaling by Wnt3a in mESCs, β-catenin enters the nucleus and binds to Tcf3, promoting its phosphorylation and degradation, thereby relieving the repression of at least a subset of Tcf3 targets and promoting stem cell self-renewal [47,48].

Seemingly conflicting reports exist on the role of Wnt signaling in promoting differentiation of mESCs and in maintaining pluripotency. These differences can potentially be explained by the vast number of transcriptional regulators that can associate with β-catenin. In addition to the TCF family, β-catenin can bind with p300, cyclic adenosine monophosphate response element binding protein (CBP), the histone methyltransferase Mll1, and numerous transcription factors of the SOX, SMAD, FOXO, and nuclear receptor families [49]. Some work suggested that although β-catenin can form a complex with p300 and with CBP, the β-catenin/CBP complex is specifically essential for mESC maintenance, whereas p300 is dispensable, and that the complex specificity can determine the activity of β-catenin [50,51]. An interesting open question is how complex specificity itself is controlled.

The mechanisms of Wnt signaling action on cell fate appear to be highly time- and context-dependent. For example, Wnt signaling activated early in mESC differentiation leads to the promotion of mesoderm development toward heart; however, the same Wnt signaling activated at a later stage of differentiation actually inhibits this fate [52]. Many of the conflicting ideas about Wnt function in differentiation and in pluripotent cells may also arise from cross-talk and interactions of Wnt with different signaling pathways that are selectively active either in ESCs or in a lineage differentiation context influenced by temporal factors.

The role of Wnt signaling in hESCs has not been fully elucidated, partly because most work has focused on hESCs under standard culture conditions, which promote a more primed cell state. Studies of cells under these conditions indicated that Wnt signaling might not be essential for hESC maintenance. However, a study looking specifically at hESCs in the naive state concluded that whereas Wnt signaling was not required for the expression of pluripotency factors in this context, it contributes to cell proliferation and colony-forming ability, and that blocking Wnt signaling led to the acquisition of a metabolic and transcriptional profile more similar to hESC in the primed state, which suggests that the role of Wnt signaling in naive hESCs is to prevent transition to the primed state [53].

THREE TRANSCRIPTION FACTORS, OCTAMER BINDING PROTEIN 4, SRY-BOX 2, AND NANOG, FORM THE CORE PLURIPOTENCY TRANSCRIPTIONAL NETWORK

The previously discussed growth factor signaling pathways that regulate pluripotency could do so through a number of different mechanisms, as outlined earlier. The most widely accepted model is that they function coordinately to inhibit differentiation-associated factors and induce expression of pluripotency-related transcription

factors. Among the many transcription factors identified and characterized as having critical roles in stem cells, a core set of three pluripotency transcription factors has been shown to form the backbone of the pluripotency gene regulatory network. These three, OCT4, SOX2, and NANOG, act together downstream of the key signaling pathways and function in part through positive feedback loops to maintain each other's expression as well as that of the wider pluripotency gene expression program. The three are thought to be central to transcriptional regulation of ESC identity because of their essential roles during early development, including in the inner cell mass (ICM), and their ability to maintain the ESC state [6,54–56]. Disruption of *Oct4* in knockout embryos and ESCs results in the inappropriate differentiation of ICM and ESC to trophectoderm, whereas *Nanog* mutants develop into extraembryonic endoderm [6,55,56]. *Sox2* loss-of-function mutants also divert to trophectoderm [54]. Notably, the phenotype of mESCs overexpressing *Oct4* resembles that of *Nanog* loss of function, forming embryonic endoderm, whereas cells with *Nanog* overexpression are highly resistant to differentiation [55]. These findings suggest a higher level of complexity in core pluripotency transcription factor functional interactions where they do not always simply work together coordinately to promote a blanket state of pluripotency and in which they can sometimes be antagonistic in specific contexts. Furthermore, they also highlight the need for exquisite fine-tuning of gene expression circuitry to promote pluripotency without completely blocking eventual differentiation capacity. Nevertheless, genome-wide analysis has revealed that these three transcription factors generally form an autoregulatory network and do so by binding to each other's promoter regions and enhancing each other's expression [57]. Furthermore, these factors regulate the expression of large numbers of downstream genes governing aspects of differentiation, cell cycle, and self-renewal [57]; and as mentioned earlier, these core factors are integral to the reprogramming process to make iPS cells.

MYC LINKS CELL SIGNALING TO PLURIPOTENCY GENE REGULATION

In addition to core pluripotency transcription factors, *c-Myc* was also found by Yamanaka initially as one of the four main reprogramming cocktail factors. Whereas *c-Myc* was subsequently determined not to be essential for reprogramming, it can potentiate efficiency of the process by one or two orders of magnitude [58]. In stem cells, MYC factors have a broader role in regulating many unique properties associated with stemness and pluripotency, including specific states of metabolism, cell signaling, cell cycle dynamics, and the epigenetic landscape. Because MYC can regulate all of these cellular functions (even if in distinct ways) in somatic and progenitor cells, where it is also expressed, it is probably not an actual "pluripotency factor" in the narrowest sense of that term. Three MYC family members have been identified and characterized: MYC, MYCN, and MYCL. In stem cells, there is a high level of redundancy between MYC and MYCN; however, knockout of both causes a loss of pluripotency and promotes differentiation [59]. Reportedly, loss of *Mycl* causes no detectable defects in stem cells and does not appear to be able to compensate for loss of the other two MYC family members [60].

MYC represents one connection point between cell signaling and the core pluripotency transcriptional network. Stem cell maintenance requires active Wnt signaling and repression of high levels of MAPK signaling. When MYC is expressed, it acts to suppress MAPK signaling by transcriptionally upregulating the ERK inhibitors, dual-specific phosphatases (DUSP2/7); it also acts to promote Wnt signaling through upregulation of WNT receptors and suppression of WNT antagonists. In mESCs, LIF is also required to activate the JAK/Stat3 pathway with *c-Myc* as a key downstream target that is activated by LIF [23].

MYC has been shown to act as both a transcriptional activator and a repressor in hESCs. In ChIP experiments, MYC is primarily associated with active histone modifications, but it also has an important role in promoting pluripotency through repressing the expression of differentiation genes including the HOX gene clusters in hESCs [61]. This is consistent with findings that loss of MYC does not have a strong effect on the expression of core pluripotency genes; the most dramatic effects instead manifest as upregulation of early differentiation genes [59]. Similarly, during reprogramming, MYC may act to suppress differentiation-specific gene expression [62]. In the repressive context in hESCs, MYC interacts with MIZ1 and repressive chromatin-modifying complexes including DNMT3A, and histone deacetylases (HDACs) [61]. The interaction between MYC and MIZ-1 appears to be antagonistic: whereas the two co-bind many targets, their effects on transcription are often in opposite directions [61]. This opposing relationship may serve to balance the contradictory forces of stem cell self-renewal and differentiation; when external signals alter the balance of MYC and MIZ-1, one force can dominate and either promote commitment to differentiation or maintain pluripotency.

MYC also influences stem cell potency in part through regulating the cell cycle, where it acts to promote S-phase progression [59]. Among MYC targets are numerous cyclins and cyclin-dependent kinase enzymes, as well as the miRNA cluster miR-17-92 [63], which targets and suppresses cell cycle inhibitors including those in the retinoblastoma family [25,64]. Because ESCs have unique cell cycle properties, it has been suggested that the effect on the cell cycle and proliferation upon loss or downregulation of MYC could trigger ESC differentiation [59].

Numerous sources of evidence indicate that whereas MYC is frequently found at gene promoters with the core pluripotency factors, some of its mechanisms of action are unique compared with other transcription factors. In fact, ChIP-microarray (ChIP-chip) and ChIP-Seq studies generally point to unique modules of gene targets for MYC compared with core pluripotency factors such as OCT4, SOX2, or NANOG. MYC also has an important role in releasing transcriptional pausing of RNA polymerase II and the transcription machinery at gene promoters, a function that is unique from core pluripotency transcriptional regulators [65].

MYC has been found to interact with a number of different epigenetic modifying enzymes in both activating and repressing conditions. These include histone acetyltransferases (HATs), HDACs, DNA helicases RUVBL-1 and -2, which are essential for ESC morphology, and histone demethylase LSD1 [66]. In addition to a role in repressing lineage specific and differentiation factors, MYC has been shown to regulate global levels of euchromatin in a number of cell types, an uncommon function for a transcription factor. This may relate to the proposed model in which MYC acts as a general activating factor at certain types of gene promoters that are already primed for gene expression by binding specific transcription factors. In this context, MYC has been shown to interact with a number of HAT complexes to promote histone H3 and H4 acetylation. Although MYC is not generally considered a pioneer factor that can bind and remodel chromatin, it has an important and early job during reprogramming to promote euchromatin by regulating global levels of histone modifications. MYC frequently binds to regions where OCT4, SOX2, or KLF4 are already bound, and then increases local euchromatin formation and transcriptional activity [67]. Thus, overall, the contributions of MYC to pluripotency are pleiotropic and mediated by a number of distinct epigenetic and transcriptomic mechanisms. MYC's importance in both stem cells and cancer further points to specific downstream pathways shared among these cell types. For instance, inhibition of differentiation-related genes, maintenance of rapid cell cycling, and an elevated metabolic state are also features of tumorigenesis, and tumorigenesis and reprogramming appear to be related phenomena [68].

A SPECIFIC EPIGENETIC PROGRAM HELPS MAINTAIN PLURIPOTENCY

The importance of chromatin modification states in regulating pluripotency is evidenced by the fact that perturbations of the expression level or function of many different epigenetic enzymes can impair stem cell self-renewal and lead to the loss of pluripotency, events that are followed by differentiation [69–72]; these enzymes are necessary for specific chromatin structural and functional states. In addition, modulation of epigenetic enzyme activity can promote cellular reprogramming. For example, the use of HDAC inhibitors, or the H3K9 methyltransferase inhibitor G9a, makes reprogramming more efficient, implicating H3K9 methylation in the process [73,74]. Other histone marks and histone-modifying enzymes are implicated as well. For example, the polycomb group complex polycomb repressive complex 2 (PRC2) is responsible for the H3K27me3 mark that acts to repress gene expression; in ESCs, this mark is found in many differentiation genes and prevents premature expression (Fig. 4.2) [75]. Loss of PRC2 complex components causes a dramatic reduction in H3K27me3 and impairs pluripotency by upregulating differentiation genes [69]. PRC2 also has a role in maintaining bivalent domains, characterized by the presence of both active H3K4me3 and repressive H3K27me3 marks together in discrete chromatin domains in developmental genes that allow for rapid activation upon commitment of the pluripotent cell to differentiation (Fig. 4.2).

Heterochromatin, marked by H3K9me3, is generally less common in ESCs than in differentiated cells. Consistent with this, the H3K9 methyltransferase G9a is important for early differentiation and development, and has been shown to recruit DNA methyltransferase enzymes Dnmt3a and Dnmt3b to downregulate pluripotency genes including *Oct4* (Fig. 4.2) [76,77]. Conversely, enzymes that remove the H3K9 methylation marks are important for maintaining pluripotency. These include Jumanji domain enzymes *Jmjd1a* (*Kdm3a*) and *Jmjd2c* (*Kdm4c*), which are upregulated by Oct4 and function to remove H3K9 methylation to maintain the pluripotency of ESCs [71].

A number of interesting differences exist between DNA methylation levels and patterns in pluripotent cells compared with differentiated cells. For example, in looking at cytosine guanine (CpG) methylation, differentiated cells frequently have enrichment of methylation in sites where there is a high density of CpG dinucleotides. In contrast, in pluripotent cells, higher methylation levels are observed in regions with lower concentrations of CpG dinucleotides [78]. These low-density CpG regions that are methylated in pluripotent cells are associated with

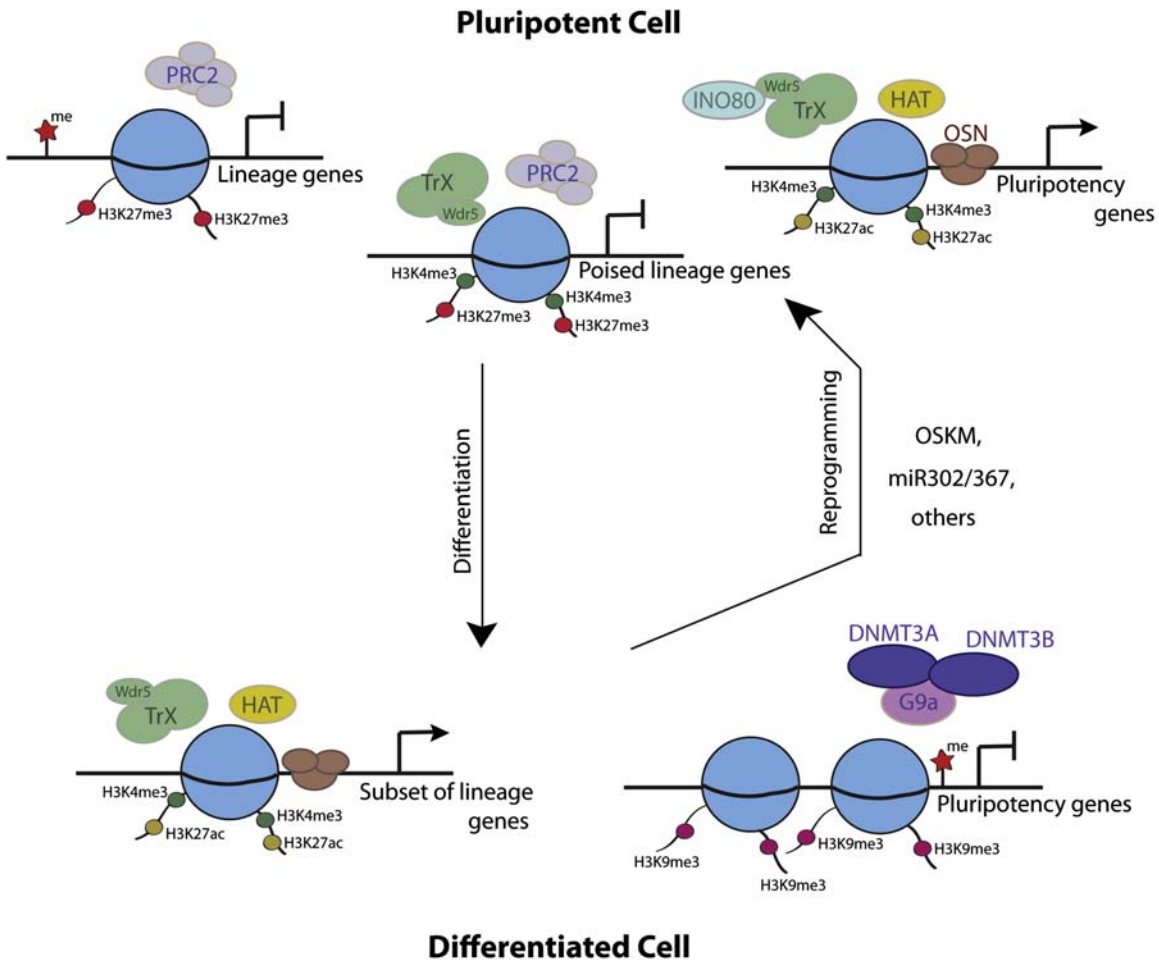


FIGURE 4.2 **Chromatin dynamics of pluripotency and stem cell differentiation.** In pluripotent cells, lineage genes are maintained in two states: repressed by polycomb complexes and DNA methylation or poised for rapid activation based on histone modifications mediated by polycomb and trithorax complexes. Pluripotency genes are maintained by octamer binding protein 4, SRY-box 2, and Nanog complex binding, as well as recruitment of active chromatin-modifying complexes. Upon differentiation, a specific subset of lineage genes is activated in each cell type by lineage-specific transcription factors that recruit activating chromatin complexes. Pluripotency genes are repressed by DNA methylation and heterochromatin associated histone marks. *HAT*, histone acetyltransferase; *OSKM*, gene expression of octamer binding protein 4, SRY-box 2, Kruppel-like factor 4, and MYC; *OSN, octamer binding protein 4, SRY-box 2, and Kruppel-like factor 4*; *PRC2*, polycomb repressive complex 2; *TrX*, trithorax; *Wdr5*, WD repeat domain 5.

bivalent chromatin marks [79] and are thought to function by being able to respond rapidly and dynamically to signals that modulate chromatin remodeling, leading to efficient activation of genes with low-density CpG methylation in their promoters.

There are three DNA methyltransferase enzymes: DNMT1, DNMT3A, and DNMT3B. DNMT1 is mainly involved in maintaining methylation over DNA replication events, whereas DNMT3A and DNMT3B modulate de novo methylation events. Deletion of any one Dnmt enzyme in mice results in embryonic or neonatal lethality [80–82], which indicates an important role in development, but mESCs deleted for one or all three Dnmt enzymes retain their pluripotency, despite losing DNA methylation globally. This suggests that in mice, DNA methylation is not required to maintain pluripotency, at least in vitro [83]. In hESCs grown under standard primed conditions, loss of both *DNMT3* enzymes does not affect stem cell self-renewal, but loss of *DNMT1A* results in cell death [84]. These findings may indicate differences in the role of DNA methylation in maintenance between human and mouse ESCs, or it may reflect the fact that hESCs are usually grown under more primed conditions, whereas mESCs are maintained in a more naive state. *DNMT3A* and *3B* are also not strictly required to generate iPS cells from differentiated cells [85].

In addition to the three DNMT enzymes, DNMT3L is a catalytically inactive DNMT protein that is highly expressed in mESCs and hESCs [86]. Its high expression in this context led to its inclusion in the 24 factors originally tested by Yamanaka for their reprogramming ability in differentiated cells. Although it does not possess enzymatic activity in itself, DNMT3L has been shown to dimerize with DNMT3A and significantly increase its enzymatic

activity [87]. DNMT3L can also interact with the N terminal tail of histone H3, and several studies have suggested that it can discriminate among different H3K4 methylations [88,89]. DNMT3L knockdown alters the methylation landscape in ESCs and affects differentiation, but it does not appear to have a strong effect on pluripotency gene expression or stem cell self-renewal [90].

DNA demethylation can occur through a series of steps in which the 5mC is converted to 5-hydroxymethylcytosine through an oxidation reaction and then further modified to 5-formylcytosine and finally 5-carboxylcytosine, which can be removed by the cell through base excision repair [91]. The methylation intermediate 5fC is often found in gene regulatory elements, including poised enhancers, and in regions associated with p300 binding [92], which suggests a regulatory function for these methylation intermediaries. The main steps of this demethylation reaction are carried out by the TET family of enzymes. TET family members *Tet1* and *Tet2* are strongly expressed in mESCs and decrease in expression during cell differentiation. Loss of *Tet1* reduces the expression of important pluripotency genes including *Klf4*, *Nanog*, and *Esrrb*, among others [93,94]. In addition, the expression of *Tet1* and *Tet2* is regulated by Oct4 [95]. During reprogramming, Tet1 has a role in removing methylation at the promoter and enhancer of *Oct4* and interacts directly with Nanog [96]. Loss of *Tet1* reduces the efficiency of iPS cell induction, whereas early activation of *Tet1* increases reprogramming efficiency [96]. Although TET proteins appear to have important roles in modulating DNA methylation in ESCs, whether they are required to maintain pluripotency is not clear: *Tet1* knockout mice survive and are fertile, and mESCs from these mice display no defects [97]. Less work has been done on TET proteins in hESCs, but knockdown of *TET2* did not affect pluripotency markers although it skewed the differentiation of hESCs toward neuroectoderm at the expense of mesoderm and endoderm [98].

The chromatin in ESCs is characterized as being more open and containing more euchromatin relative to heterochromatin, compared with more differentiated cell types. Therefore, it is unsurprising that histone modifications enriched in euchromatin and associated with more active transcriptional states have also been shown to have an important role in maintaining the pluripotent state by promoting euchromatin. Histone acetylation neutralizes the charge affinity between DNA and histone proteins, leading to more open chromatin that is found at active chromatin domains [99]. That a variety of HDAC inhibitors consistently increase reprogramming efficiency [74] also indicates that open, active chromatin is an important feature of pluripotency. Trithorax group complexes are responsible for the H3K4me3 mark that is strongly enriched at active promoters, and core trithorax complex member *Wdr5* is most highly expressed in pluripotency [70]. *Wdr5* expression decreases as differentiation progresses, and it can aid in reprogramming [70]. It is also a downstream target of Oct4, Sox2, and Nanog [70]. WD repeat domain 5 protein interacts directly with OCT4 and chromatin remodeler INO80 to increase activation of self-renewal genes [100].

In addition to the interaction of cell signaling molecules with transcription factors, there are also extensive interactions between cell signaling and chromatin itself. For example, JAK signaling results in phosphorylation of tyrosine 41 on histone H3, leading to reduced affinity of heterochromatin protein 1α binding at pluripotency genes and promoting gene expression [101]. The chromatin remodeling factor Brg1 acts in Stat3 target genes in pluripotent cells [102]. The embryonic stem cell Brg1/hBrm associated factor complex, an SWI/SNF chromatin remodeling complex that contains Brg1 as a subunit and has a crucial role in mESCs, has also been shown to be an integration point for multiple signaling pathways and transcriptional regulators. Not only does it associate with Oct4, Sox2, and Nanog, it binds with Stat3 and Smad1, linking chromatin remodeling to both the LIF and BMP signaling pathways [102–104].

MICRORNAS INTEGRATE WITH CELL SIGNALING AND TRANSCRIPTION FACTORS TO REGULATE STEM CELL PROLIFERATION AND DIFFERENTIATION

Noncoding RNAs represent an additional level of regulation of gene expression and have an important role in stem cell maintenance as well as cellular differentiation. MicroRNAs, a class of noncoding RNAs, are short single-stranded RNA molecules that are usually 20–30 nucleotides in length and function by targeting a specific set of mRNA transcripts, interfering with their transcription. The most common mechanism for miRNA processing involves a series of cleavage steps mediated by enzymes Drosha and Dicer [105]; the resulting miRNA is incorporated into a protein complex known as the RNA-induced silencing complex [106].

Relatively few miRNAs are expressed in ESCs compared with differentiated cells [107–109]. Despite this, studies have identified several clusters of miRNAs that are highly enriched in ESCs. For instance, the miR302 cluster is selectively expressed in both mESCs and hESCs compared with differentiated cells, whereas mESCs also express the

miR290 cluster and hESCs express the miR17 family, and miRNAs from the chromosome 19 miRNA cluster and miR-371-373 cluster (thought to be orthologous to mir290 in mESC) [110–112]. Core pluripotency transcription factors Oct4, Sox2, and Nanog can bind the promoters of most of these ESC-enriched miRNAs [113]. The importance of these miRNAs to ESC maintenance was demonstrated by deleting miRNA processing enzymes Drosha and Dicer. *Dicer* knockout resulted in embryonic lethality in mice [114], which indicated an important role for miRNA in development. In addition, in mESCs, knockout of *Dicer* or the Dicer binding partner DiGeorge syndrome critical region gene 8 caused proliferation defects and prevented proper differentiation; however, stem cell self-renewal appeared to be preserved [115–117]. Similarly, in hESCs, knockdown of *DICER* or *DROSHA* caused reductions in cell proliferation [118]. These results indicate that miRNAs function in ESCs to regulate the cell cycle: in particular, the shortened G1-S transition that uniquely characterizes stem cells. More broadly, it is hypothesized that pluripotency is facilitated by short cell cycle gap phases and that gap phases represent specific windows of opportunity for stem cells to initiate the differentiation process.

Another interesting aspect of miRNA is their ability to contribute to reprogramming during iPS cell formation. Expression of miR290 cluster members or several other miRNAs can increase the efficiency of reprogramming [119–121], whereas knockdown of *Dicer* or *Drosha* reduces efficiency [119]. Reportedly, reprogramming was completely inhibited by the deletion of the miR302 cluster in human fibroblasts [122]. ESC-expressed miRNAs have also been shown to influence cell signaling; miR290 members can repress Wnt signaling inhibitor Dkk1 in mESC [123], and mir302 targets Lefty, an inhibitor of the TGFβ/Nodal signaling pathway in hESCs [124].

CHROMATIN STRUCTURE DETERMINES REGULATORY ACTIVITY OF TRANSCRIPTION FACTOR BINDING TO PLURIPOTENCY GENES

Two-dimensional views of chromatin (such as strictly linear perspectives on transcription factor control of nearby target genes) do not reflect its unique three-dimensional (3D) structure accurately. The major roles of 3D chromatin structure in pluripotency have come into focus. Regulation of chromatin structure and the formation of chromatin loops are essential aspects of pluripotency; the proper expression of members of the cohesin and mediator complexes is required for stem cell maintenance, and the preexisting chromatin structure in somatic cells has been shown to have a strong influence on the reprogramming efficiency of these cells into iPS cells [125]. In fact, the expression of transcriptional factors that regulate reprogramming and their binding to gene promoters of pluripotency targets such as *Oct4* is not enough on its own to activate gene transcription; the proper chromatin structure must also be in place. In the case of *OCT4* in hESCs, it has been shown that in addition to the binding of transcription factors such as OCT4 and NANOG, activation requires a downstream enhancer to be in close proximity to the promoter, and that this structure depends on the mediator and cohesin protein complexes [126].

The intersection of transcription factor binding and chromatin structure is also evident in long-range interactions that are present at the *Nanog* locus. In mESCs and iPS cells, it has been shown that mediator and cohesin are responsible for approximately 40% of the observed long-range genomic interactions with the *Nanog* locus, and that Med1 and Smc1a interact directly with pluripotency and reprograming factors Oct4, Sox2, Klf4 [127]. A separate study showed that tethering Nanog to a region of the genome in mESCs was sufficient to induce a number of chromatin loops between pluripotency genes, which artificially created a Nanog binding site [128]. In addition, Klf4 is required for cohesin complex recruitment to *Oct4* and for the resulting loop between enhancer and promoter [129].

Together, these results suggest that pluripotency and reprograming factors are involved in mediating chromatin looping and in the recruitment of cohesin and mediator complexes to gene targets where chromatin looping has essential roles in pluripotency. From some of these studies, it is also evident that chromatin looping at pluripotency genes tends to occur before gene activation. For example, during the reprogramming process, it is striking that pluripotency-specific genomic interactions of the *Nanog* locus occur several days before activation of *Nanog* expression [127]. These data support a model in which reprogramming factors bind and act to recruit cohesin and mediator complexes to restructure the chromatin, forming loops that place enhancers and promoters in proximity, and group coexpressed genes together, before these factors then can activate gene expression.

CONCLUSIONS

The molecular basis of pluripotency is a complex coordination of extracellular and environmental factors with intracellular signal transduction and transcriptional regulation via specific epigenomic events. We have seen significant leaps in understanding of how signaling cascades converge upon core transcriptional circuitry to coordinate

maintenance and induction of pluripotency. Furthermore, understanding of the intricate mechanisms through which signaling pathways create the multitude of cell types and tissue lineages remains paramount to understanding basic human development as well as to manipulating and controlling lineage specification for the purposes of regenerative medicine. The rapid advent of induced pluripotency by reprogramming differentiated cells into an embryonic state through cocktails of transcription or chemical factors has opened significant doors to the concept of personalized regenerative medicine. Understanding the fundamental mechanisms of this process may ultimately provide us with unprecedented control to reprogram somatic cells into iPS cells or directly to any desired cell type for the purpose of cell transplantation. Toward that goal, new technologies such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR−associated protein 9 gene editing and pluripotent stem cell−based organoids are likely to accelerate new discoveries further.

List of Acronyms and Abbreviations

BMP Bone morphogenic protein
EpiSC Epiblast stem cell
FGF Fibroblast growth factor
GSK3β Glycogen synthase kinase 3β
HDAC Histone deacetylase
hESC Human embryonic stem cell
iPS cell Induced pluripotent stem cell
LIF Leukemia inhibitory factor
MEK mitogen activated protein kinase/extracellular signal−regulated kinase
mESC Mouse embryonic stem cell
OCT4 Octamer binding protein 4
PRC2 Polycomb repressive complex 2
SOX2 SRY-box 2
TCF3 Transcription factor 3
TGFβ Transforming growth factor β

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CHAPTER

5

Scarless Wound Healing: From Experimental Target to Clinical Reality

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INTRODUCTION

Though scarring is the normal outcome of wound healing in adults, little is talked about its long-term consequences. For instance, because skin and other organs scar rather than regenerate after injury, several physiologic sequelae often occur. After bowel surgery, the visceral and parietal peritoneum develop dense scar tissue known as adhesions, which cause postoperative bowel obstructions [1]. After traumatic injury to soft tissue, ligaments, and tendons, scarring can cause contractures that limit movement and cause functional restriction [2]. Scarring in the nervous system results in loss of function because neuronal connections are destroyed [3]. In the cornea, scarring limits visual acuity [4]. Cardiac myocytes are also severely affected by scarring, as seen by the number of arrhythmias associated with impaired conduction after infarct [5]. The only exceptions to the rule of fibrosis after injury in adults are found in bone, oral mucosa, and hepatic tissue, which are capable of partial regeneration [6,7].

The most significant phenotype in skin scar formation can be found in burn injuries [8,9]. Approximately 500,000 patients in the United States undergo medical treatment for burn injuries annually; over one-third of patients have burns that exceed 10% of the total body surface area (American Burn Association Burn Incidence Fact Sheet, 2016). In addition, many of these patients are children, a population that is particularly vulnerable to the negative physical and psychological effects of scarring; 10,000 children are permanently disabled each year from burn injury and fibrosis [8–12]. Perhaps the most dramatic consequence of burns are hypertrophic scars, which afflict up to 70% of burn patients in their lifetime. These dense, fibrous scars can limit range of motion severely and can negatively affect quality of life through pain, itching, and irritation rendered from the scar tissue itself [13]. All of this, of course, does not account for the long-term psychological impact caused by delayed reintroduction to society after hospitalization, partly from disability but also from issues related to confidence and cosmetic appearance [13].

As mentioned, not only normal scars cause problems for adult and pediatric patients. Issues with underhealing and overhealing also cause significant morbidity. Nonhealing chronic ulcers and excessive fibroproliferative scarring are the most common examples of dysfunctional wound healing, each of which is prevalent in certain pediatric and adult populations. Often, dysfunctional wound healing will cause pain, functional restriction, and severe psychological outcomes [14,15]. In patients with chronic illnesses, dysfunctional wound healing usually falls into the category of "underhealing". In this situation, wounds fail to heal for numerous reasons, including infection, impaired blood flow, severe malnutrition, and inadequate wound care. These patients have become an increasing concern, particularly as the population ages and more health care resources are allocated to treat chronic diseases and their associated complications.

Diabetic patients are a dramatic example of the burden of chronic wounds on society. The following statistics, obtained from the Centers for Disease Control and Prevention's 2014 National Diabetes Statistic Report, illustrate the magnitude of diabetic nonhealing wounds: 29 million people in the United States have diabetes, which makes diabetic patients approximately 10% of the US population. In addition, 65% of diabetic patients have at least one other

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co-occurring illness. In 2010, 73,00 nontraumatic lower limb amputations were performed in patients with diabetes. A total of 23% of all patients with diabetes have foot problems ranging from numbness to amputations. From 25% to 50% of all hospital admissions for diabetes are for nonhealing ulcers, which are the cause of most nontraumatic extremity amputations performed in the United States each year [16].

On the other extreme, excessive healing is also a serious clinical burden. Pathological scarring causes hypertrophic scars and keloids. Whereas hypertrophic scars eventually abate with management and time, keloids continue to grow as a type of soft tissue tumor that may persist for life after excision [17]. These scarring processes cause functional impairment and symptoms such as burning, itching, and pain. Ultimately, hypertrophic scars and keloids are difficult to treat medically or surgically, particularly because uniform treatment algorithms do not exist [18].

As is noted from these examples of normal and dysfunctional wound healing, the burden of wound healing is substantial in the United States and on patients. As such, research in the field is urgently needed to help patients regain functional abilities and limit morbidity after injury. Here, we discuss the normal mechanism of adult wound healing and explore the remarkable ability of fetal skin to regenerate. Current and future therapeutics will be discussed, with the hope of highlighting the potential of regenerative healing.

ADULT SKIN

Anatomy of Adult Skin

Adult skin is made up of two layers, the epidermis and dermis. The epidermis is a type of keratinizing stratified squamous epithelium that has five distinct layers, each characterized by the level of keratinocyte maturation. Keratinocytes originate from a thin sheet of stem cells in the basal epidermis and migrate to the surface over 4 weeks to become soft keratin, which eventually sloughs off.

Epidermal appendages, which are epithelial structures that extend intradermally, are an important source of cells for reepithelialization in skin wound healing. Epidermal appendages include sebaceous glands, sweat glands, apocrine glands, and hair follicles. Appendages can extend deep into the dermis or through the dermis into the subcutaneous tissue/hypodermis.

The hair follicle is an important appendage composed of an external outer root sheath attached to the basal lamina that is contiguous with the epidermis. The hair follicle also contains a channel with a hair shaft. Together, the hair follicle and its attached sebaceous gland are called the pilosebaceous unit. The base, or bulb, of the hair follicle contains a committed but proliferating progenitor cell population, as well as the matrix encasing the dermal papilla. The dermal papilla contains specialized mesenchymal cells. From this region, the hair shaft and its channel will grow.

Sweat glands (or eccrine glands) produce sweat, a mixture of water and salts, which functions to cool the body by evaporation. The sweat gland is distinct in that it contains a coiled intradermal portion that extends into the epidermis by a relatively straight distal duct. These glands, too, have an important role in normal skin, as well as physiologic homeostasis.

Below the epidermis lie two distinct layers of the dermis: the more superficial papillary layer and the deeper, more fibrous reticular layer. The papillary dermis is highly vascular, sending capillaries (dermal papillae) superficially. The reticular dermis contains densely packed collagen fibers and tends to be less vascular, except where sweat glands and hair follicles traverse through the tissue. Reticular dermis is also rich in elastic fibers and contains some macrophages, fibroblasts, and adipose cells that participate in skin wound healing [19] (Fig. 5.1).

Adult Wound Healing and Scar Formation

Adult wound healing is traditionally described as a sequence of temporally overlapping phases: inflammation, proliferation, and remodeling. Disruption of the vascular network within cutaneous wounds results in platelet aggregation and the formation of a fibrin-rich clot, which protects from further extravasation of blood or plasma. Aggregation of platelets initiates the coagulation cascade [20–22]. In addition to providing hemostasis, platelets modulate fibroblast activity through degranulation and secretion of multiple cytokines and growth factors, such as platelet-derived growth factor (PDGF), platelet factor 4, and transforming growth factor β1 (TGF-β1). These growth factors and cytokines remain elevated throughout the process of normal wound healing [23,24].

Largely under the influence of platelet-derived inflammatory molecules, neutrophils and monocytes initiate their migration to the wound. Because of the high concentration of neutrophils in circulation, these cells are the first to enter the area of injury and quickly reach high concentrations, becoming the most dominant influence in early wound healing. Neutrophils primarily produce degradative enzymes and phagocytose foreign and necrotic material, but they also produce vascular endothelial growth factor (VEGF), tumor necrosis factor α, interleukin 1 (IL-1),

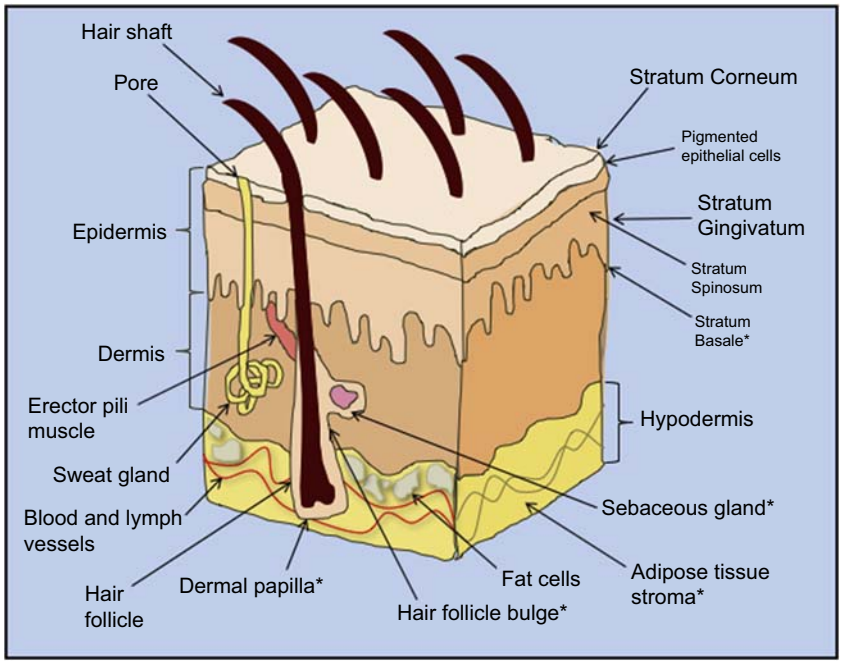

FIGURE 5.1 **Normal skin anatomy.** Normal skin anatomy is composed of two layers, the epidermis and dermis, with a layer of subcutaneous fat known as the hypodermis below. Contained within the dense array of epithelial cells and extracellular matrix are many specialized structures including sebaceous glands, sweat glands, and hair follicles. In wound healing, normal tissue homeostasis, and tissue engineering, skin stem cells have an important role. Areas where resident adult stem cells are located are indicated by an *asterisk*. Not pictured are interfollicular epidermal stem cells.

and other growth factors that assist in wound healing [25]. Interestingly, studies show that neutrophil infiltration is not essential to normal healing, which demonstrates one of many redundancies in the repair process [26].

The level of inflammation in a wound ultimately depends on the presence or absence of infection. In the presence of infection, neutrophils continue to be active in high concentration throughout later stages of wound healing, leading to further inflammation and fibrosis [22]. In the absence of infection, neutrophils greatly diminish activity by day 2 or 3, as monocytes increase in number in response to both extravascular and intravascular chemoattractants. Monocytes differentiate into macrophages that bind to the extracellular matrix (ECM), which induces phagocytosis and allows for debridement of necrotic cells and fractured structural proteins. During the late inflammatory phase, tissue macrophages release cytokines and scavenge dead neutrophils, then elevate macrophages as the dominant leukocyte in the wound bed. In contrast to neutrophils, studies on tissue macrophage and monocyte-depleted guinea pigs demonstrated macrophages to be essential to normal wound healing through their stimulation of collagen production, angiogenesis, and reepithelialization [27]. However, like neutrophils, if macrophages persist in the wound environment, the result is excess scar formation. Under these circumstances, macrophages produce cytokines that activate fibroblasts to deposit excessive amounts of collagen [28] (Fig. 5.2).

The presence of macrophages in the wound marks the transition between the inflammatory phase and the proliferative phase of wound healing, which begins around day 4–5 after injury. Granulation tissue begins to form and is described as a loose network of collagen, fibronectin, and hyaluronic acid embedding a dense population of macrophages, fibroblasts, and neovasculature. During the deposition of granulation tissue, macrophages and fibroblasts move into the wound space as a unit while new blood vessels sprout from adjacent exposed endothelium [22].

The rate of granulation deposition depends on many factors, including the interaction between fibronectin and fibroblast integrin receptors [29]. Fibroblasts in the wound bed deposit collagen and a proteoglycan-rich provisional matrix, a process that is stimulated by TGF-β1 and TGF-β2 in adult wounds. Studies have shown that exogenous administration of these molecules leads to increased collagen and inflammatory cells at the wound site, which potentially implicates this subfamily of growth factors as a source of overactive scar formation [30,31].

During the proliferative phase of wound healing, which occurs from approximately day 5 to day 14 after wounding, collagen is deposited more densely in the wound. Once a threshold level of collagen is reached, collagen synthesis and fibroblast accumulation are suppressed by an unknown negative-feedback mechanism [32]. The balance

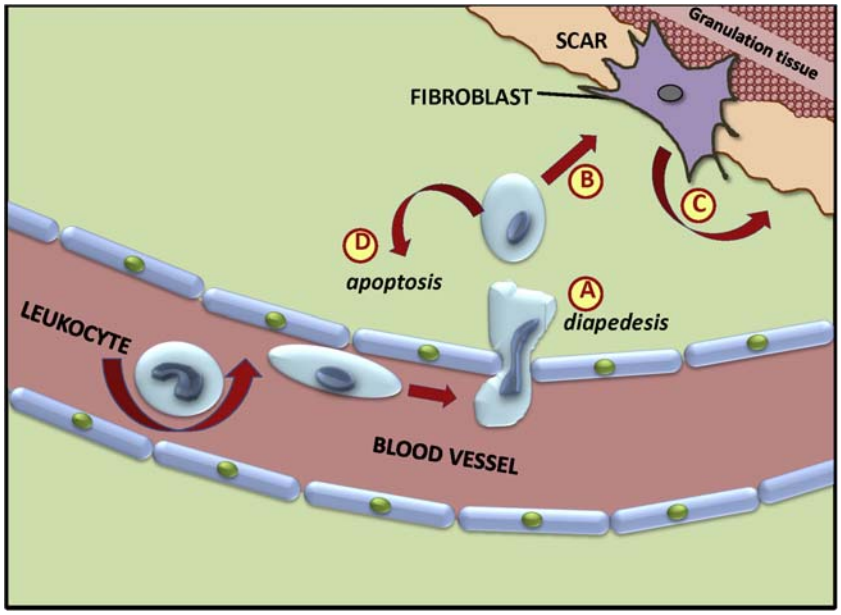

FIGURE 5.2 **Inflammatory cell recruitment to the site of tissue damage.** Therapeutic intervention aimed at dampening the immune response could target any of the steps along the pathway of inflammatory cell recruitment. (A) Leukocytes in blood vessels adjacent to the site of tissue damage emigrate through the vessel wall by diapedesis and (B) migrate to the site of tissue damage in response to chemotactic signals. Inflammatory cells activate resident fibroblasts and attract other bone marrow–derived cells to the wound, where the repair outcome is (C) scar formation. After acting at the wound site, the activated repair cells disperse, differentiate, or (D) apoptose, thus ending the repair response.

of collagen synthesis and degradation is controlled by collagenases and tissue inhibitors of metalloproteinases. When this negative feedback does not occur appropriately, pathological scars form with the deposition of densely packed, disorganized collagen bundles [22].

Reepithelialization begins in the first 24 h after wounding, with the goal of creating a new protective, natural skin barrier. Initially, basal keratinocytes at the border of the wound, which under normal circumstances are linked together by desmosomes, detach from each other and the ECM and migrate laterally to fill the void in the epidermis. Through this process, keratinocytes are exposed to serum for the first time. As they are subjected to new and increased levels of inflammatory cytokines and growth factors, keratinocytes are signaled to further migration, proliferation, and differentiation [33].

Neovascularization also occurs during the proliferative phase and is influenced by multiple cytokines, circulating endothelial progenitor cells, and the ECM [34]. The formation of new blood vessels can also be stimulated by lactic acid, plasminogen activator, collagenases, and low oxygen tension [22]. New blood vessels in the wound bed bud and grow at an exceptional rate during skin wound healing, eventually giving the wound bed adequate oxygenation and its characteristic pink hue. Once apoptotic pathways become active again as the granulation tissue matures, angiogenesis eventually desists and the numerous new blood vessels reduce to only a few major tributaries [35].

The maturation phase of wound healing consists of collagen remodeling beginning during the second week of healing. At this point, fibroblasts differentiate into myofibroblasts, which are characterized by greater expression of smooth muscle actin and the ability to contract wounds. Fibroblasts eventually decrease in number from the proliferative phase, and the scar tissue becomes less vascular and paler as vessels involute [36].

Scar tissue finally gains tensile strength as collagen cross-links increase during remodeling, largely owing to the action of myofibroblasts. Maturation involves the replacement of the initially randomly oriented types I and III collagen by predominantly type I collagen organized along the lines of tension. Eventually the process completes; however, scar tensile strength will never reach that of unwounded skin [37] (Fig. 5.3).

Fibroproliferative Scarring

Fibrosis is defined as "the replacement of the normal structural elements of the tissue by distorted, nonfunctional, and excessive accumulation of scar tissue" [38]. Many medical problems are linked to excessive fibrosis, in addition to a number of deaths each year in the United States and across the globe [37,39]. As previously

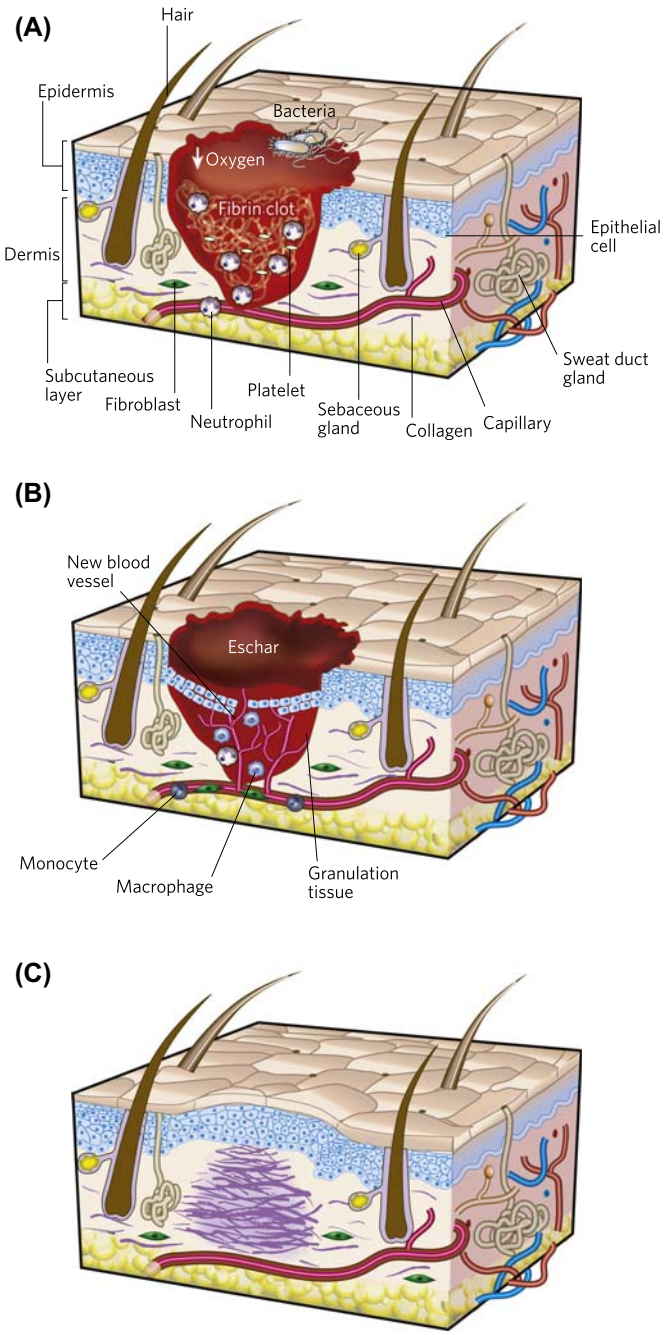

FIGURE 5.3 **Classic stages of wound repair.** There are three classic stages of wound repair: inflammation (A), new tissue formation (B), and remodeling (C). (A) Inflammation. This stage lasts until about 48 h after injury. Depicted is a skin wound at about 24–48 h after injury. The wound is characterized by a hypoxic (ischemic) environment in which a fibrin clot has formed. Bacteria, neutrophils, and platelets are abundant in the wound. Normal skin appendages (such as hair follicles and sweat duct glands) are still present in the skin outside the wound. (B) New tissue formation. This stage occurs about 2–10 days after injury. Depicted is a skin wound at about 5–10 days after injury. An eschar (scab) has formed on the surface of the wound. Most cells from the previous stage of repair have migrated from the wound and new blood vessels now populate the area. The migration of epithelial cells can be observed under the eschar. (C) Remodeling. This stage lasts for a year or longer. Depicted is a skin wound about 1–12 months after repair. Disorganized collagen has been laid down by fibroblasts that have migrated into the wound. The wound has contracted near its surface and the widest portion is now the deepest. The reepithelialized wound is slightly higher than the surrounding surface and the healed region does not contain normal skin appendages. *Reproduced with permission from Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. Nature 2008; 453(7193):314–21.*

mentioned, excessive fibroproliferative scarring occurs when the mechanisms of wound healing fail to respond to inhibitory cues at any one of the phases of wound healing. In essence, abnormal scar formation is an excess accumulation of unorganized collagenous ECM. Although the appearance of scars is often unpredictable, several factors influence the severity of scarring. These include not only genetics but also tissue site, sex, race, age, magnitude of injury, and wound contamination. Generally, skin sites with thicker dermal tissue tend to heal with thicker scars. Also, estrogen is believed to promote scarring; premenopausal women have denser scar tissue than do postmenopausal women. Patients with darkly pigmented skin are also more prone to thicker scarring, as are young people. Larger, deeper, and more contaminated wounds tend to produce larger resultant scars, as well [40–44].

Keloids

Over months to years, some scars will develop benign but locally aggressive tumors known as "keloids" [45]. This extreme example of fibroproliferative scarring is characterized by "cauliflower" nodules that extend well beyond the area of original injury and continue to grow, sometimes into significant masses [45]. Symptoms can range from irritation and pain to severe disfigurement and functional restriction owing to the appearance, size, color, and location of lesions [17,18,28,39,46–48]. In addition to being disfiguring, keloids tend not to regress. Instead, they can continue to grow slowly over many years, and their growth rate tends to correlate with symptoms. The most common areas affected by keloids are upper-body sebaceous areas, whereas the extremities are less commonly involved [49]. Aside from being involved in areas with a high density of sebaceous glands, tension may have a role in their development over time [45].

Histologically, keloids are characterized by thick, large, closely packed bundles of disorganized collagen. In addition, the scar tissue is colonized by mast cells, eosinophils, plasma cells, and lymphocytes with a stark absence of macrophages [45]. Unlike hypertrophic scars, keloids have nodules in the mid to deep dermis containing densely packed collagen with a few myofibroblasts. Mucin is deposited focally in the dermis, and hyaluronic acid expression is confined to the thickened, granular/spinous layer of the epidermis. Finally, an amorphous and unidentified mucopolysaccharide surrounds the dense collagen bundles in keloidal scars that is not found in other scar tissue types [18].

Interestingly, in keloids, more than one mechanism of dysfunctional healing may be at play. Fibroblasts in keloids tend to respond abnormally to wound healing growth factors, secreting collagen, elastin, and fibronectin with little response to inhibitory compounds. Moreover, the ability for fibroblasts to proliferate in keloids is increased. In addition, dysfunctional apoptosis has been observed as keloidal wounds enter the remodeling phase of wound healing [50]. It is possible that this is partly the result of central scar ischemia and ongoing release of VEGF, hypoxia inducible factor 1α, and other growth factors that promote proliferation [17,18,28,39,46–48]. Finally, although a number of therapies exist for the treatment of keloids, including surgical excision, laser therapy, cryotherapy, and chemotherapy, keloids remain remarkably resistant and recur over time [51–54]. Because of these features, it is possible that keloid tumor cells are mutated skin progenitor cells which are known to display a similar resilience [55] (Fig. 5.4A).

Hypertrophic Scars

The incidence of hypertrophic scars, another type of fibroproliferative scar, is significantly higher than keloids. This is partly because of the large number of young adults with initially hypertrophic scars that eventually regress with age (35%) [56]. Hypertrophic scars are raised, similar to keloids; however, they are usually no more than 4 mm from the surface of skin. Visually, hypertrophic scars are erythematous or brown-red in color, but they can become pale over time. Also, hypertrophic scars will sometimes regress spontaneously. Yet, also unlike keloids, which are predominantly over skin containing sebaceous glands, hypertrophic scars usually occur on extremity joints such as the elbows and knees [48].

Histologically, hypertrophic scars are characterized by the expansion of collagen bundles that are fine, well-organized, and parallel to the epidermis. Hypertrophic scars do not have the nodal collagen bundles seen in keloids, but they have islands of hypercellularity in the deep dermis that upon closer inspection contain aggregates of fibroblasts, collagen, and neovasculature. Mucin is absent and hyaluronic acid is a major component of the papillary dermis [18,49].

The purported mechanism of hypertrophic scar formation is increased and ongoing collagen deposition with decreased collagenase activity. In addition, fibroblasts in hypertrophic scars are more likely to assume a myofibroblast phenotype, which may contribute to collagen deposition and scar contracture without addressing scar remodeling. Because 70% of burn victims are affected, initial wound depth may have a significant impact on the development of hypertrophic scars, because they appear to be most common in patients with delayed healing or

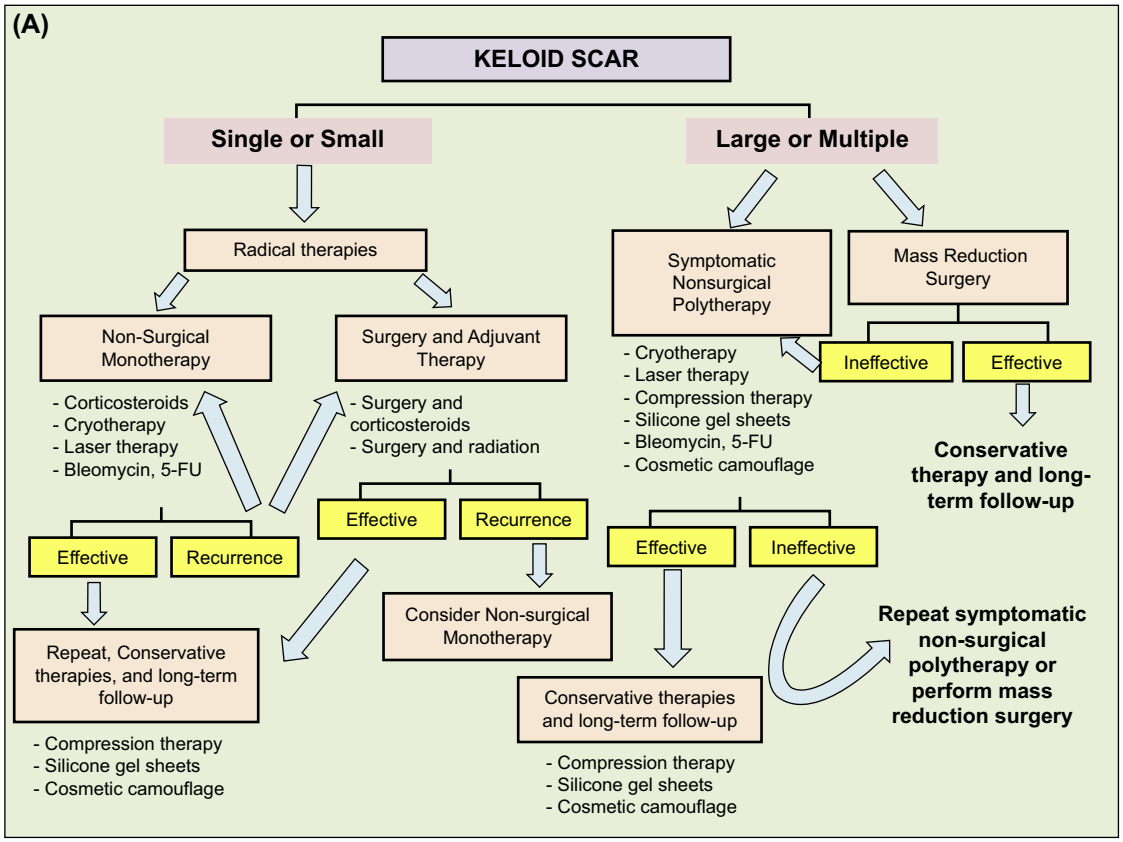

FIGURE 5.4 **Scar reduction strategies: algorithms for (A) keloids and (B) hypertrophic scars.** *Modified from Ogawa R. The most current algorithms for the treatment and prevention of hypertrophic scars and keloids. Plast Reconstr Surg 2010; 125(2):557–68.*

a prolonged inflammatory phase. In deeper and larger wounds, fibroblasts assume a myofibroblast phenotype, which renders them less capable of reducing type I collagen in scars during the remodeling phase [56] (Fig. 5.4B).

Underhealing: Chronic Skin Ulcers

Many types of chronic nonhealing dermal ulcers exist, such as pressure ulcers, diabetic lower-extremity ulcers, and venous stasis ulcers. Besides causing pain and disfigurement, chronic ulcerations often lead to infection as well as amputation. In patients with chronic ulcerations such as those who have diabetes, often the single most important life-saving measure is limb salvage to preserve mobility and function [57]. In those who are immobile, pressure ulcers predominate, as seen by their commonality among debilitated or institutionalized patients, those with spinal cord injuries, and patients with cerebrovascular infarcts. The total cost of wound care per hospitalization for pressure ulceration is estimated to be $130,000, a figure expected to grow as the population ages [58].

Interestingly, nonhealing wounds have distinct histologic hallmarks too, as do keloid and hypertrophic scars have their own set of unique histologic hallmarks. In chronic ulcers, there is excessive neutrophil infiltration, and wounds seem to remain in the inflammatory phase. Likely, the abundance of neutrophils is responsible for the lingering inflammation seen in chronic ulcers. As neutrophils release enzymes, such as collagenase (matrix metalloproteinase [MMP] 8), connective tissue is digested as fast as new matrix is deposited [59]. Neutrophils also release elastase, an enzyme known to destroy PDGF and TGF-β, which are important growth factors for normal wound healing [60]. The environment of chronic ulcers is also known to contain an abundance of reactive oxygen species that damage healing tissue [61]. The result of this nonhealing mechanism is a wound that not only fails to heal but perpetuates its existence.

FETAL SKIN

Development of Fetal Skin

The skin's superficial layer, the epidermis, is derived from surface ectoderm, whereas the dermis is generated from mesenchyme. The epidermis starts as a single layer of ectodermal cells covering the embryo, which begins to emerge at gestational day 20 in humans. In the second month, cell division takes place, at which time the periderm (epitrichium) emerges as a thin superficial layer of squamous epithelium overlying the basal germinative layer. Over the next 4–8 weeks, the epidermis becomes highly cellular. New cells are produced in the basal germinative layer and are continuously keratinized and shed to replace cells of the periderm. Together, these cells are part of the vernix caseosa, a greasy white film that covers fetal skin. In addition to desquamated cells, the vernix caseosa contains sebum from sebaceous glands. With sebum and desquamated cells mixed together, this slippery substance serves as a protective barrier during gestation and facilitates passage through the birth canal at delivery.

Replacement of the periderm continues until the 21st week, at which point the periderm has been replaced by the stratum corneum. Through a series of stages of differentiation, the epidermis stratifies into four layers by the end of the fourth month, including the stratus germinativum (derived from the basal layer), the thick spinous layer, the granular layer, and the most superficial stratum corneum. By the time these four layers emerge, interfollicular keratinization has begun and the epidermis has developed buds that become epidermal appendages. Melanocytes of neural crest origin have also invaded the epidermis, synthesizing melanin pigment that can be transmitted to other cells through dendritic processes. By the end of the 21st week, the fetal epidermis has many of the components that it will maintain into adulthood. After the 21st week, the dermis begins to mature from a thin and cellular structure to a thick and fibrous one. By 24 weeks of gestation, fetal skin matures and thickens to become histologically distinct from its embryonic beginnings [62].

Fetal Scarless Wound Repair

Whereas adult wounds heal with fibrous tissue (scarring), early-gestation fetal skin wounds heal scarlessly. Fetal wounds are capable of healing with restoration of normal skin architecture and preservation of both tissue strength and function. This observation has been confirmed in multiple animal species, including mice, rats, rabbits, pigs, sheep, and monkeys. The mechanisms responsible for fetal scarless wound healing are intrinsic to fetal tissue and are independent of environmental or systemic factors such as bathing in sterile amniotic fluid, perfusion with fetal serum, or the fetal immune system [63–65]. To support this point, studies in which human fetal skin

that was transplanted subcutaneously into the dorsolateral flank of athymic mice healed without a scar further suggested that the scarless wound phenotype depends on characteristics intrinsic to fetal tissue [66].

Ultimately, scarless fetal wound repair outcomes depend on two factors: the gestational age of the fetus and the size of the wound. Excisional wound healing studies performed on fetal lambs showed that at a given gestational age, larger wounds healed with an increased incidence of scar formation. Likewise, the frequency of scarring increased with increasing gestational age [67]. Since the publication of these studies, transitional periods have been found for humans (24 weeks' gestation) [68], rats (between gestation days 16.5 and 18.5) [64], and mice [69]. Extensive research has been dedicated to determining the culprit for the shift to adult wound healing. Eventually, instead of depositing bundles of ECM in a normal basket-weave pattern, organisms begin to heal breaches in skin integrity with scarring.

Interestingly, as fetuses develop and enter the early period of scar formation, the wound phenotype has been described as a "transition wound." At this point, the repair outcome is tissue that retains the reticular organization of collagen characteristic of normal skin but is devoid of epidermal appendages [70]. The skin does not truly regenerate, but the dermis does not form a scar.

In addition, although the fetal ECM was once thought to be inert, evidence suggests that it is a dynamic structure that has a pivotal role in cellular signaling and proliferation. The fetal ECM is now known to be a reservoir of growth factors essential to development [71]. The fetal ECM also has a structural protein composition different from that of adult skin. For example, the collagen content of the ECM changes as the fetus ages, starting with a relatively high type III to type I collagen ratio and shifting to the type I collagen-predominated adult phenotype. Another structural difference between fetal and adult ECM can be found in the hyaluronic acid content. Hyaluronic acid, the negatively charged, extremely hydrophilic, nonsulfated glycosaminoglycan of the ECM, has been shown to be in higher concentration in the ECM during rapid growth processes such as cellular migration and angiogenesis. In vitro studies show that hyaluronic acid can cause fibroblasts to increase the synthesis of collagen and noncollagen ECM proteins [72]. During adult repair, hyaluronic acid initially increases dramatically, and then decreases from days 5 to 10, after which the concentration remains at a low level. Interestingly, this hyaluronic acid profile is not the case in fetal wound ECM, in which the hyaluronic acid level remains high. Similar to the concentration of type III collagen, the ECM hyaluronic acid content decreases from the fetal to the postnatal period [63].

The concentration of other substances such as decorin, fibromodulin, lysyl oxidase, and MMPs further sets the fetal ECM apart from the adult ECM [71]. These substances are proteoglycan ECM modulators that have a role in the development and maturation of collagen. Lysyl oxidase cross-links collagens whereas MMPs degrade collagen. In addition, decorin content and the expression of lysyl oxidase and MMPs increase as fetal tissue matures. Fibromodulin modulates collagen fibrillogenesis and has been shown to bind and inactivate TGF-βs. Fibromodulin decreases with gestational age, paralleling the shift from scarless fetal wound healing to scarring adult repair [73] (Fig. 5.5).

REGENERATIVE HEALING AND SCAR REDUCTION THEORY

Targeting the Inflammatory Response

Initial research into the mechanisms responsible for scar formation led investigators to focus on the inflammatory phase of wound healing as a target for reducing the incidence and magnitude of scar formation. This choice was based on observations that regenerative wound healing is replaced by scarring as the immune system develops in the embryo [65]. Interestingly, many studies have shown that reduction of inflammation in postnatal skin wounds correlates with reduced scarring [74].

One example of reduced inflammation and scarring can be found in enhanced healing in mice devoid of mothers against decapentaplegic homolog 3 (Smad3), a protein known to transduce TGF-β signals. These mice exhibited more rapid reepithelialization and decreased inflammation (blunted monocyte activation) [43]. In another example, PU.1 null mice devoid of functional neutrophils and macrophages heal wounds over a time course similar to that of their wild-type counterparts but exhibit scar-free healing similar to embryonic wound healing [75]. These two studies support the contention that the inflammatory response may be deleterious to normal wound repair by contributing to increased fibrosis. Experiments performed on athymic mice [74] and experiments involving antisense downregulation of connexin43, a protein involved in gap junctions and inflammation, support these findings [74,76]. Furthermore, other studies have provided evidence that wound inflammatory cells from the circulation

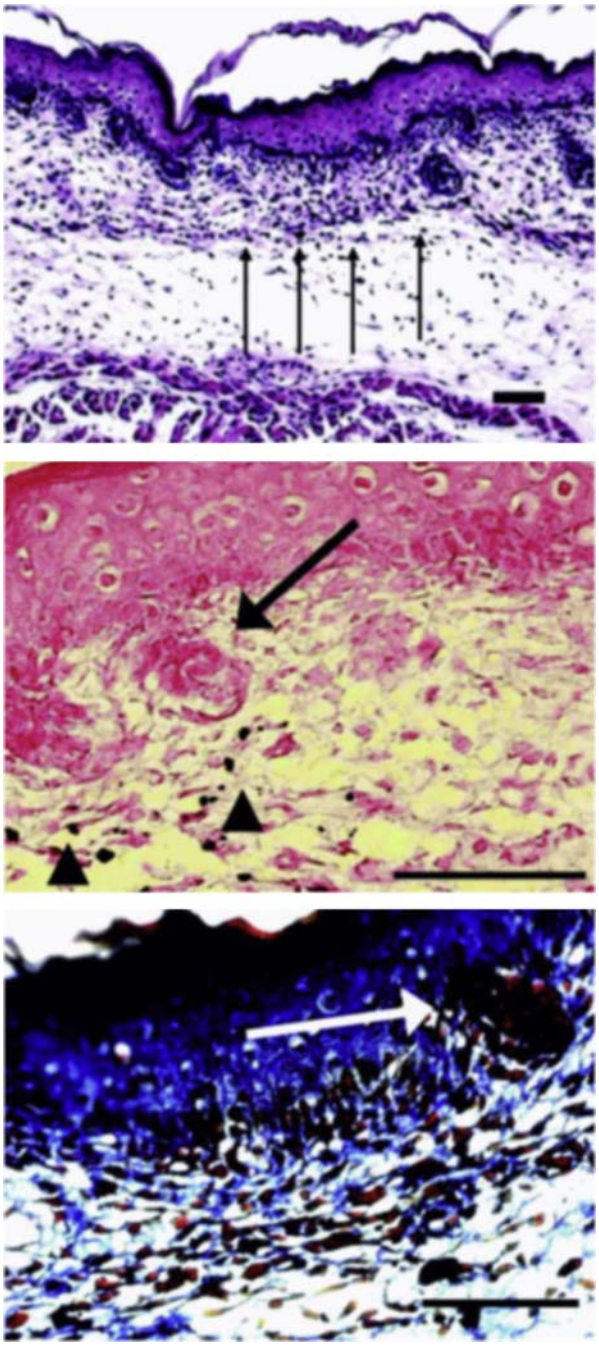

FIGURE 5.5 **Wound histologic sections at 48 h after injury in embryonic day 17 fetuses.** India ink was injected at the time of injury. (A) Low-power magnification reveals complete reepithelialization and a mild increase in the number of inflammatory cells present (arrows) (hematoxylin and eosin; original magnification, ×100; bar = 100 μm). (B) High-power view shows India ink (arrowheads) collected around regenerating hair follicles (arrow) (eosin; original magnification, ×400; bar = 25 μm). (B) Mallory trichrome shows a fine reticular dermal collagen pattern. A white arrow shows a hair follicle (Mallory trichrome; original magnification, ×400; bar = 25 μm). *Reproduced with permission from Colwell AS, Krummel TM, Longaker MT, Lorenz HP. An in vivo mouse excisional wound model of scarless healing. Plast Reconstr Surg 2006; 117(7):2292–96.*

produce signals that either directly or indirectly induce collagen deposition and granulation tissue formation, which increase scarring [77].

Although this research points to the inflammatory phase of wound healing as a cause of scar tissue formation, studies have provided evidence that the inflammatory phase and scarring might not be as directly linked as previously believed. Cox-2, an enzyme involved in prostaglandin production, is a mediator of inflammation. Two studies

show conflicting evidence regarding the effect of Cox-2 inhibition; one study reported decreased scar formation and the other claimed no difference in wound healing or scar formation [78,79]. Likewise, a study that transiently induced neutropenia in mice accelerated wound closure but failed to show a difference between collagen content in neutrophil-depleted wounds compared with wild-type controls [80].

Although other possible mediators of scar formation exist, the inflammatory response remains a major target for ongoing research aimed at preventing or reducing the appearance of scars. As Stramer et al. illustrated, many points exist at which interventions could dampen the inflammatory response. The first target could be leukocytes, at any point as they migrate (1) through the vessel wall from the bloodstream, (2) from outside the vessel to the wound, or (3) as they transmit a signal to fibroblasts, inducing the fibrotic response. A second target could be the fibroblasts themselves, and interventions could be designed to block the action of these cells as they respond to leukocyte signaling [81].

An interesting development in the scar theory was the discovery of heterogeneous fibroblast populations, with certain fibroblasts being responsible for the entirety of scar collagen production. *Engrailed-1*, a gene for a homeobox protein, was used to trace a lineage of fibroblast appearing at the transition from regenerative to scarring phenotype in the dorsal skin of fetal mice, and was also found to deposit all skin scar collagen in adults. This discovery may have uncovered the elusive element behind scar formation and may offer a specific cellular target to achieve regenerative healing. Also, along with the discovery of *Engrailed-1* positive fibroblasts was the discovery of specific inhibitors of these fibroblasts, offering already a potential therapy to achieve reliable scar reduction. Through targeting scar fibroblasts rather than inflammatory cells, perhaps other phases of wound healing, such as the proliferative and remodeling phases, may also be investigated having potential therapeutic benefit [82].

Cytokines and Growth Factors

Transforming Growth Factor β Superfamily

Though there is an abundance of knowledge pertaining to the TGF-β pathway, the development of clinical tools targeting this growth factor have largely arrested. The TGF-β superfamily includes several proteins, the most important are which are TGF-β1, TGF-β2, and TGF-β3; all of them have been shown to influence adult wound healing [83]. These cytokines are secreted by keratinocytes, fibroblasts, platelets, and macrophages, which act to influence their own and other cell populations to migrate into the wound bed [84]. The TGF-β superfamily has also been implicated in matrix remodeling and collagen synthesis [85]. Partly, this comes from evidence that TGF-β1 activates myofibroblast differentiation to influence wound contraction and the synthesis of collagen and fibronectin in granulation tissue [86].

Investigators have compared the TGF-β isoform profiles of fetal and adult skin. Results showed that injured fetal epidermis contains a greater amount of TGF-β3, derived from keratinocytes and fibroblasts, and less TGF-β1 and TGF-β2, derived from degranulating platelets, monocytes, and fibroblasts, compared with healing adult skin [87,88]. Since this cytokine profile was discovered, isoforms TGF-β1 and TGF-β2 have generally been thought to be pro-fibrotic, whereas TGF-β3 is thought to support scarless healing [84]. Discovery of the relative ratios of these isoforms prompted experiments aimed at mimicking the embryonic profile, using antibody neutralization of TGF-β1 and TGF-β2 and treatment with exogenous TGF-β3 [89]. Although antibody neutralization of TGF-β1 or TGF-β2 has no effect on wound healing, experiments showed that antisense RNA knockdown of TGF-β1 does in fact reduce scar formation [90]. Likely, the length of time that TGF-β1 is neutralized over the course of wound repair influences scarring, with longer neutralization needed for greater influence on scar reduction [84]. In addition, the coordinated actions of TGF-β1 and TGF-β3 in wound healing may be more complicated and necessary than originally hypothesized, because alterations in the ratio of TGF-β1 and TGF-β3 fail to produce significant results [91].

Interestingly, clinical trials with recombinant TGF-β3 failed to demonstrate diminished scarring. Avotermin, which received acclaim and investment in early clinical trials, failed to meet its Phase III end points [92]. Since then, Avotermin and its alternative were abandoned, perhaps highlighting the importance of integrating TGF-β with tissues, cells, or other cell signaling molecules. Now, with the expansion of wound healing biomaterials, the TGF-β superfamily is being targeted as a therapeutic to be delivered via biomimetic scaffolds [93]. Engineered as well as dermal composite scaffolds are emerging as potential therapies, with some promising in vivo studies [94].

Connective Tissue Growth Factor

Connective tissue growth factor (CTGF), like many other growth factors in wound healing, is capable of influencing fibroblast differentiation and inflammatory cell migration [95]. It is also considered profibrotic by a mechanism related to TGF-β, and through its influence on fibroblasts to deposit scar ECM matrix [95], as evidenced by its

increased expression in scleroderma patients [96]. CTGF is a TGF-β target gene that is activated by Smad proteins after TGF-β binds to and activates its receptors. Once activated, this cysteine-rich matricellular protein binds to integrin and proteoglycan receptors activating pathways such as wingless type (Wnt), bone morphogenic protein, VEGF, and TGF-β [97], which highlights the redundancy in many important wound healing signaling cascades.

Adult fibroblasts have higher expression of CTGF than fetal fibroblasts, which makes this an attractive therapeutic target. Studies on fetal fibroblasts stimulated by TGF-β show increased expression of CTGF, suggesting that scarless fetal repair may be partially a result of lower CTGF expression [98]. Following this evidence, CTGF was found to have a positive vulnerary effect in diabetic wound healing, accelerating the time to wound closure in diabetic foot ulcers [95]. In a rabbit ear model, hypertrophic scarring improved using messenger RNA (mRNA) antisense inhibition of CTGF [99]. Like TGF-β, CTGF may be involved in too many overarching signaling cascades to influence wound healing by solitary treatment with recombinant or inhibitory compounds, although its predominant temporal influence in wound healing may be limited to the early inflammatory phases [97].

Vascular Endothelial Growth Factor

There are four isoforms of VEGF, VEGF A through D. Keratinocytes, fibroblasts, and macrophages all produce VEGF, which is thought to be one of the main regulators of angiogenesis and vasculogenesis in wound healing. VEGF acts through two receptors in endothelial cells, VEGF-R1 and VEGF-R2.

In adult wound healing, VEGF increases over time and has been associated with angiogenesis [71]. Also, direct and indirect targets of VEGF can affect wound healing [100,101]. However, through studies on fetal rats, scarless healing has an increase in VEGF expression three times higher than what is observed in late-gestation fetal wounds [73]. This work suggests that increased VEGF expression is partially responsible for the accelerated wound healing that occurs early in gestation, but ultimately its role may not be in regulating scarless wound repair.

Fibroblast Growth Factors

Embryonic wounds contain lower levels of fibroblast growth factors (FGFs), growth factors involved in skin morphogenesis [88]. The expression of FGFs, including keratinocyte growth factors 1 and 2, increases as the fetus ages, which suggests that these growth factors are profibrotic, similar to CTGF and TGF-β [102]. Many isoforms have been studied, including FGF-5, which doubles in expression at birth, FGF-7, which multiplies more than sevenfold at birth, and FGF-10, which doubles at the transitional period [71]. In general, downregulation of the FGF isoforms occurs during scarless wound healing, whereas the opposite is true during adult wound healing, which suggests that FGF upregulation is likely partially responsible for scar formation or at least managing cell differentiation and proliferation [71].

Studies suggest that FGFs, as well as Wnts, also have a role in organizing and differentiating embryonic organs [103–106]. Moreover, FGFs may be stored in the ECM by binding to proteoglycans, becoming a reservoir during tissue injury, when serum proteases cleave FGFs into functionally active isoforms and begin their signaling cascade [104]. In injured murine skin, FGF-9 initiates wound-induced hair follicle neogenesis through a feedback loop including two different fibroblast subtypes, the Wnt2a/β-catenin pathway, and FGF-10 [107]. Although studies in electroporation may provide an example of dermal appendage regeneration through ECM maintenance [108], these studies have not been verified, which makes FGF-9 the only single-cell signaling molecule capable of restoring some normal skin architecture during adult skin healing.

Platelet-Derived Growth Factor

Like FGF, PDGF has been identified as a profibrotic growth factor. Adult wounds contain high amounts of PDGF, whereas this growth factor is virtually absent in embryonic wounds. However, this is largely the result of inhibition of platelet degranulation in embryonic wounds [88]. Experiments involving the administration of PDGF to fetal wounds show that this growth factor induces scarring through increased inflammation, fibroblast recruitment, and collagen deposition [109].

Wingless Type Signaling

Wnts are a critical regulatory cell signaling molecule existing in the form of secreted glycoproteins. Wnts, which are usually tethered to a lipid moiety such as a palmitate tail, usually have limited diffusion capacity and function in local tissue environments [110]. In embryos, Wnts have a major role in axial differentiation. In adults, Wnt responsive cells are found in all major tissue types and origins, which suggests Wnts continue to have important roles in adult tissue organization [111]. In adult skin, Wnt-responsive cells can be found in the epidermis and dermis. By expert opinion, Wnt signaling cascades in adult stem cells are thought to be responsible for ongoing cell fate, spatial

recognition, and cell polarity, the importance of which is often understated [111]. In addition, specific Wnt proteins such as Wnt2a may be involved in the cell-to-cell communication necessary for specialized dermal structures to develop, such as new hair follicles in wounded adult skin, as was previously discussed [107].

Wnt expression in fetal tissue is usually higher than in adult tissue at baseline and often gradated [111,112]. With wounding, the expression of Wnts in fetal dermal tissue, as with many other growth factors and inflammatory cells, changes very little [112]; instead, the overwhelming number of organizational growth factors and *Hox* gene products leads to scarless tissue regeneration. In adults, Wnts increase during skin wound repair. However, their role in skin wound healing is less certain because Wnts (1) function mostly as a signal transducer and (2) are difficult to study [112]. Ultimately, Wnts are a promising although enigmatic choice for studying wound healing. Their only foreseeable barriers in clinical translation are appropriate dosing and delivery modalities, because Wnts also have a role in cancer development [106,107].

Interleukins

The interleukins (ILs) are a class of cytokines involved in activating the inflammatory cascade; they are frequently targeted as wound healing adjuvants. IL-8 stimulates neovascularization and attracts neutrophils. IL-6 is produced by adult fibroblasts in response to stimulation by PDGF, and then activates macrophages and monocyte chemotaxis. With an insult to skin integrity, IL-6 and IL-8 rapidly increase expression to facilitate recruitment of circulating inflammatory cells to the wound. This elevated expression is maintained over 72 h during adult repair but is suppressed after 12 h during scarless fetal repair. Early fetal fibroblasts express lower levels of both IL-6 and IL-8 than do their adult counterparts at baseline and in response to PDGF stimulation. Therefore, these proinflammatory cytokines are thought to promote scarring. In addition, studies on the administration of IL-6 to fetal wounds induces scarring, which further supports this theory [65,113].

However, IL-10 is thought to be antiinflammatory, based on its antagonism of IL-6 and IL-8. Evidence for this comes from fetal embryonic day (E)15 skin in IL-10 knockout mice grafted to syngeneic adult mice. Incisional wounds on these skin grafts showed scar formation, whereas similar wounds in 15-day gestation wild-type skin grafts on adult wild-type mice healed scarlessly. These results suggest that IL-10 is essential for scarless fetal healing owing to its ability to dampen the inflammatory response [65,113]. In a supporting study, administration of an IL-10 overexpression adenoviral vector reduced inflammation and induced scarless healing in adult mouse wounds [114].

CURRENT THERAPEUTIC INTERVENTIONS

No currently available therapy can induce postnatal regenerative healing. Although many therapeutic interventions are used to reduce scar formation, research has not adequately demonstrated efficacy or safety for many of these treatments, because of small treatment groups and a lack of well-designed studies. However, the following treatments are used clinically to reduce scarring symptoms and scar formation.

Topical and Intralesional Corticosteroid Injections

Corticosteroids are commonly used to treat symptomatic scars; triamcinolone is the most commonly used agent. The mechanism of action is multifactorial. The inflammatory response is globally decreased, which secondarily decreases collagen synthesis and increases collagen degradation. Corticosteroids also inhibit fibroblast proliferation and TGF-β1 and β2 expression by keratinocytes [115,116]. Although 50−100% symptom improvement has been reported, studies are limited by a lack of appropriate controls and poor design [115,117].

The use of corticosteroids is limited by reported adverse consequences in 63% of patients. These effects include delayed wound healing, hypopigmentation, dermal atrophy, and scar widening [117]. Based on successful studies combining corticosteroid injections with 5-fluorouracil therapy and laser therapy, polytherapy may be the best method with which to use steroids, because lower dosing and fewer adverse effects occur [117,118]. Overall, an agent with global reduction in cell protein synthesis and proliferation is not ideal for achieving tissue regeneration.

5-Fluorouracil

In the treatment of fibroproliferative scars, 5-fluorouracil (5-FU) has been used since the 1990s, with some promising clinical results [119]. This therapy, which represents a middle ground between the low side-effect profile of

silicone gel sheeting and the morbidity of surgery, has emerged as a promising alternative for patients who wish to avoid surgery or who have failed conventional treatment algorithms. 5-FU has gained some negative attention because of its use as a chemotherapeutic agent. When given systemically, it has potent side effects though a long-established safety profile. Its mechanism of action involves inhibiting the synthesis of the pyrimidine thymidine, thus inhibiting DNA synthesis in dividing cells and inevitably causing apoptosis [119].

In glaucoma and other proliferative diseases (such as keloids), 5-FU offers promising results when administered locally. When used alone, 45–78% of patients saw improvement in their scar appearance. However, when combined with triamcinolone, the efficacy of local administration improved scar appearance in 50–96% of patients [119]. It may also be combined with surgery to reduce fibroproliferative scar recurrences. Unfortunately, most clinical trials recruit small numbers or are confined to populations inherently at risk for fibroproliferative scarring [120,121]. Further research analyzing the efficacy of 5-FU in scarring is needed, but given its relatively benign local side effects and demonstrated benefits, it should remain an alternative for patients with recalcitrant scarring disorders [18,119,121–123].

Imiquimod

Originally marketed as a treatment for verruca, actinic keratosis, and basal cell skin cancer, imiquimod may be used in low-morbidity settings for the treatment of hypertrophic scars and keloids. This Toll-like receptor-7 agonist functions by stimulating dermal inflammatory cells to secrete interferons, ultimately recruiting activated leukocytes to skin when applied topically. There, the effect is mostly mediated by the immune system. This medication represents an exciting, minimally toxic, and targeted method of immune modulation with few systemic side effects, and mostly blistering or skin irritation as the major local side effect.

A few small, randomized controlled trials evaluated the efficacy of imiquimod in the treatment of acute surgical incisions in the breast [124] and in minor dermatologic surgeries [124]. However, the most trials failed to show a difference in scar appearance and potentially may have worsened outcomes by potentiating inflammation [124]. In the case of already formed keloids and hypertrophic scars, imiquimod could be tested in select patients wishing to try nonsurgical therapies, but ultimately this product does not appear to have a role in acute wound healing.

Laser Therapy

In the 1990s, pulsed dye laser therapy emerged as a potential treatment for fibroproliferative and acute scars [125,126]. Like other disorders of aberrant proliferation, the mechanism proposed involves destruction of new blood vessels to limit, at a minimum, the erythematous appearance of some scars [126]. The idea behind targeting fibroproliferative scars with laser treatment comes from the principle that vascularity is partially responsible for their erythematous appearance. Although some case studies showed positive results in the treatment of keloids, the ischemic mechanism of keloid progenitor cell differentiation and proliferation is concerning [127].

Laser therapy has relatively few adverse effects (hyperpigmentation in 1–24% of patients and transient purpura in some). However, more research is needed to support its efficacy.

Bleomycin

Another potent chemotherapeutic being used in dermal fibroproliferative disorders is bleomycin. This antibiotic has profound antibacterial, antiviral, and antitumor activity through DNA strand scission [128]. The exact mechanism of action is not entirely understood, but it is generally accepted that bleomycin induces cell death by forming complexes with iron and other metals that generate free radicals that eventual cleave both double- and single-stranded DNA, as well as degrade RNAs [128]. In resistant hypertrophic scars, keloids, and warts, bleomycin has been used off-label by dermatologists via intradermal injections [129]. Similar to other off-label use products, there are few clinical trials testing its efficacy [129]. However, if monitored closely with an experienced practitioner, it may be effective and safe. Side effects from treatment with bleomycin may be as minor as skin irritation, to as severe as skin necrosis and eschar formation [129]. When giving systemically, bleomycin notoriously causes pulmonary and skin fibrosis in humans and mice [130]. As such, it is often used to induce both conditions in an effort to create mouse models of pulmonary fibrosis and scleroderma [130]. Like other chemotherapeutic agents exploited in wound healing, development of a targeted therapy would be more attractive.

Silicone Gel Sheets

Maintaining tissue hydration in the base of wounds has long been known to improve outcomes in acute and chronic wound healing. Silicone, in either sheets or gels, emerged in the 1980s as a potential vulnerary agent by providing a hydrating barrier to open wounds. For decades, silicone has been used in deeper tissues, in the form of breast implants or as an interface for matrices used in hernia repair and large tissue defects; which highlight its safety [131,132]. Silicone gel sheets are hypothesized not only to hydrate wounds but also to inhibit collagen deposition and downregulate TGF-β2. Its development came from a series of studies that implicated dehydration of deep dermal fibroblasts as the mechanism for inducing scar collagen production. This therapy has been studied for both treatment and prophylaxis of excessive scarring [133]. Initial studies showed conflicting results in terms of efficacy [134,135], requiring further study. A review emphasized the weak evidence surrounding the use of silicone gel sheets in the treatment of keloids and hypertrophic scars [136]. However, silicone gel sheets will likely continue to be used because they are a noninvasive treatment with few adverse effects. In clinical practice, silicones are usually found as a component to a complex matrix or as a topical therapeutic dressing for chronic wounds [132]. Although the efficacy is unknown, they are safe to continue using and investigating.

Pressure Dressings and Negative-Pressure Wound Therapy

Despite being in clinical use since the 1970s [137], pressure dressings have not been validated by experimental trials to be efficacious in prophylaxis or in the treatment of scars [138]. These treatments may be efficacious in reducing the appearance of a scar if used in polytherapy, but further investigation is warranted. Pressure earrings have been used in earlobe keloid excisions but have not been shown to eliminate recurrence.

Pressure is also used in the form of negative-pressure wound therapy. Most surgeons are familiar with this technique, in which a vacuum is applied to an open wound (chronic or acute) to activate mechanotransducers in cells and potentiate cell proliferation and eventual wound closure by secondary intention [139]. The application of negative pressure in wound therapy appears to be ever increasing, with promising results as a treatment for acute wounds [140], chronic wounds [141], burns [142], and for the closure of large contaminated incisions [143]. Many modifications have been made to the vacuum system, such as the application of silicone sheets or foam pads, which ultimately make minor improvements in the overall technology.

Radiation Therapy

Radiation therapy can also be used as an adjunct to surgical excision in the treatment of keloids. Mechanistically, it is thought to decrease collagen production by reducing fibroblast proliferation and neovascular bud formation. Radiation therapy is most effective for recurrent keloids if a single dose is given within 24 h of surgical excision, but studies have attempted short courses of radiation after healing with positive results, as well [144]. Radiation treatment appears to decrease recurrence rates after surgical excision from 45–100% to 16–27% of patients [145–147]. The magnitude of difference from these studies clearly highlights their limited power.

One limitation of radiation therapy is that standardized dosing, fractionation, time period, and frequency after surgical procedures has not been developed. Reish et al. reported good results in treating recurrent keloids after surgery with 300–400 Gy in three to four fractions or 600 Gy in three fractions [138]. However, like many other studies in wound therapy, large, randomized, controlled studies are required to evaluate the role of radiation further in wound healing. Ideally, therapy that may cause oncogenesis should be avoided. Given the morbidity of radiation, this is not a popular method to reduce scar tissue formation.

Cryotherapy

Cryotherapy has been studied in conjunction with surgical excision to treat keloids and hypertrophic scars. Many of these studies are limited by small sample size and poor controls, but the largest study reported a 79.5% response rate with 80% reduction in scar volume [148,149]. Cryotherapy is thought to decrease collagen synthesis and mechanically destroy scar tissue. Side effects include hypopigmentation and depressed atrophic scar formation [150]. This therapy can be used as an adjunct to surgery or as monotherapy, but studies question its efficacy in large recalcitrant keloids [151].

Extracellular Matrix Substitutes and Scaffolds

As the extracellular matrix is researched, it is becoming increasingly apparent that once regarded "inert" structural proteins such as collagen, vimentin, fibronectin, hyaluronic acid, proteoglycans, and glycosaminoglycans are in fact functional and can regulate cell growth [152]. With this discovery, scaffolds mimicking the ECM targeting acute or dysfunctional wound healing have emerged at a rapid rate. The most basic ECM scaffolds are made of single ECM compounds such as collagen or hyaluronic acid, at times integrated in bilayers with synthetics. Examples include Hyalomatrix, Promogran, and Biocol [152]. Acellular dermal matrices, human amniotic membranes, and porcine intestinal mucosa, which once were used for large incision closures and hernias, are also emerging in the market as wound healing adjuvants. These, too, can be combined with a number of additional therapies, from impregnated pharmaceuticals to cultured stem cells [153]. Generally, results are promising in the treatment of chronic and diabetic wounds [152]. However, large randomized controlled trials have not been performed comparing different ECM substitutes.

Tension Offloading

An important field of study in wound healing focuses on mechanical forces. There are multiple examples of pressure, suction, or stretch that result in rapid cell proliferation, as is seen with vacuum-assisted wound closure and pregnancy [139,154]. In adult wound healing, tension initiates a signaling cascade leading to the proliferation of local cells typically in a symmetric pattern. Both keratinocytes and fibroblasts have mechanosensors imbedded in their cell membrane. These sensors, with other molecules that bind to the ECM such as integrins, are ion channels that open only when stretched [155]. In the case of fibroproliferative wounds, this may be an inciting factor leading to aberrant cell growth [139]. In fetal wound healing, the loss of dermal architecture leads to the deposition of actin protrusions that will contract and recruit local cells to close gaps in tissue and eventually regenerate lost structures [156]. This is perhaps the most stark difference in wound healing between prenatal and postnatal organisms, in which similar filaments and structures are involved but ultimately are used in entirely different ways [156].

In surgical patients, tension can have a severe impact on wound healing. On the back, chest, sternum, and tibia, incisions naturally stent open and can be predisposed to prolonged healing, infection, and dehiscence whereas areas with low tension and redundant tissue, such as the eyelid, heal with minimal scarring. A clinical trial tested an external tension offloading device known as "embrace®" in acute scar revision [157]. Patients achieved significantly diminished scarring with its use.

Surgery

Remodeling is a process that can last 1–2 years. During this time, scars can lose their dark pigmentation, flatten, soften, and contractures can lessen. Because scars can often behave unpredictably, surgery is usually reserved until after this period has passed. There are many options for surgical treatment for scarring, including excision with direct closure, local skin flap coverage, or more extensive vascular flap coverage. These treatments are generally considered before surgical treatment or as an adjunct. For fibroproliferative scars, surgical treatment is usually a simple excision, with or without flap closure, depending on the size of the resulting defect. In chronic wounds, burns, and pressure ulcerations, surgery may consist of creating local or pedicled tissue flaps, split-thickness skin grafts, or repeated surgical debridements to encourage healthy wound healing.

FUTURE THERAPEUTIC INTERVENTIONS

Growth Factors and Cell Signaling Molecules

As mentioned earlier, targeting individual cell signaling molecules has not translated to effective clinical treatments. In the case of recombinant TGF-β, clinical trials were arrested when the treatment failed to affect wound healing [92]. Monotherapy with solitary ECM components also usually leads to some treatment effect, but none that regenerates normal dermal architecture. Studying the signals influencing cell polarity and differentiation, however, has been more fruitful. FGF-9 is being developed as a therapy for hair loss [107] whereas its target, Wnt2a, may prove to be important in generating new hair placodes. WNt3a has already proved to be valuable in regenerating tissues, and in a mouse model of tissue regeneration, full-thickness excisional wounds taken from the ears closed whereas

placebo treatments did not [158]. It is likely that if signaling cascades are targeted specifically via receptor agonists and antagonists, polytherapy will be needed to circumvent redundancies in the inflammatory pathway and signals leading to collagen deposition. These few examples represent the most promising monotherapies currently in use.

Targeting Gap Junctions and Connexins

Gap junctions are hypothesized to function during wound repair by transferring injury signals from cell to cell, coordinating the inflammatory response, mediating wound closure, and regulating scar tissue formation in response to injury [46,159–161]. Many connexins (Cx) are present in the skin; the most extensively studied connexin is Cx43, which is expressed in both the epidermis and dermis [76]. Cx43 has decreased expression at the wound edge by the first 1 or 2 days after injury [159]. During wound repair, increased phosphorylation of Cx43 by protein kinase C may cause decreased gap junctional communication through a decrease in unitary channel conductance. This inhibition then initiates the injury-related response by the involved cell, which recognizes injury via reduced cell-to-cell communication [162].

When Cx43 antisense oligonucleotides were applied to mouse skin wounds, decreased inflammatory cell infiltration, fibrotic tissue deposition, and accelerated wound healing were observed [163]. Other studies showed that transient inhibition of Cx43 decreases scarring after burn injury in wild-type mice and increases reepithelialization after burn injury in human diabetic patients [163,164]. To further support these data, Cx43 knockouts have accelerated wound closure [165] and decreased collagen type I synthesis in the presence of chemicals that uncouple communication between cells. Interestingly, these treatments did not affect the levels of collagen type I mRNA [46]. Based on these data, the application of lithium chloride, a substance known to enhance signal propagation through gap junctions, produced the opposite effect: enhancing the deposition of granulation tissue, increasing open wound closure time, and increasing scar [166].

Given the strong correlation between Cx inhibition and improved wound healing, other therapies aimed at blocking signal transduction from cell to cell are under investigation. For example, a group at the Medical University of South Carolina synthesized a membrane permanent peptide containing a sequence designed to inhibit interaction of the ZO-1 protein with Cx43. This peptide, known as ACT1 peptide, decreases the rate of channel organization in gap junctions [167]. Through further investigation, researchers found that this peptide interacts with more than one portion of Cx43 and enhances cutaneous wound healing through decreased inflammation and scarring [160]. The advantage of this novel protein is that overall Cx43 expression is not altered. Moreover, the expression of other genes is not directly altered, unlike with antisense therapy and gene knockdown modalities.

As with the TGF-β superfamily, several commercial companies are attempting to develop Cx-related scar reduction therapies. These therapies include Cx43 antisense-based gene therapy and ACT peptide bioengineering [167], which are in the early stages of testing and will not be available for some time.

Other Drugs and Biologics

Additional strategies increase the expression of intrinsic antiscarring molecules at the wound site, including fibromodulin, hyaluronic acid, and hepatocyte growth factor [168–170]. Other approaches include treatment with inhibitors of MMP [171], inhibitors of procollagen C-proteinase [172], inhibitors of dipeptidyl peptidase IV enzymes [173], as well as treatment with angiotensin peptides [174]. Adenovirus-p21 overexpression has also been linked to scar reduction [175].

Stem Cells

True skin regeneration at sites of injury has not been accomplished by single molecule–specific therapy. As such, regenerative repair may require a cell-based approach. Stem cell therapy, with the ability to differentiate cells into various necessary cell types, is a promising approach to regenerative repair (Fig. 5.6).

Embryonic Stem Cells

Embryonic stem (ES) cells are those that may be isolated from the embryo and possess both pluripotency and the capacity for unlimited self-renewal. The discovery of ES cells was the result of work in the 1970s involving transplanting embryonic cells into ectopic sites, which inevitably resulted in teratomatous tumor formation [177]. The discovery of specific embryonic cells that self-renew indefinitely in culture and can generate an entire organism

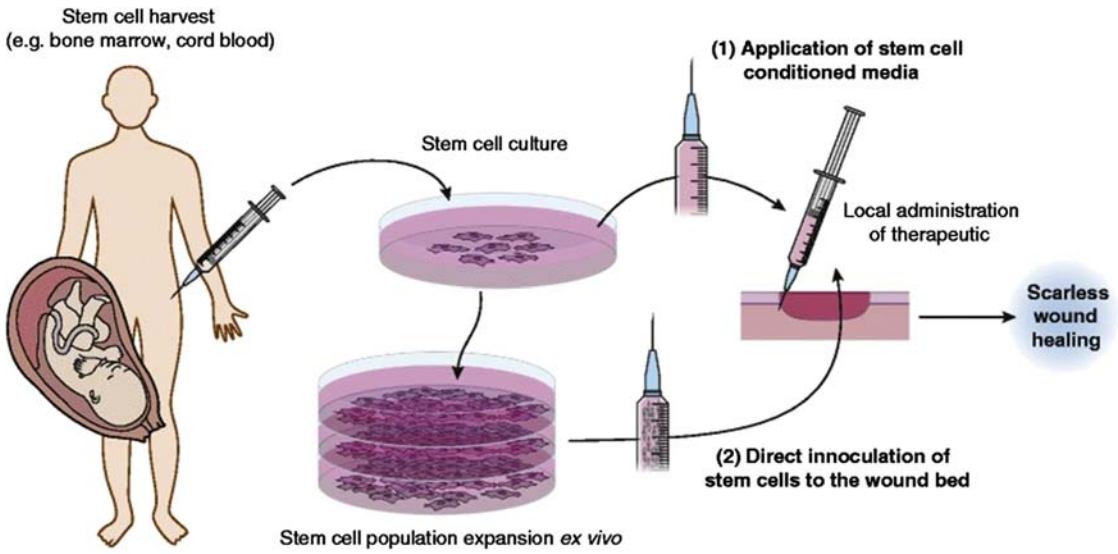

FIGURE 5.6 **Stem cell–based therapies to promote scarless wound healing.** Representation showing general principles of two cell-based therapeutic methodologies: (1) application of stem cell–conditioned media, and (2) direct application of stem cells to the wound bed. The poor survivability of mesenchymal stem cells (MSCs) transplanted to the wound bed has prompted the development of other novel therapies that take advantage of the paracrine mechanisms of action of these cells. Application of conditioned media from umbilical cord blood–derived MSC culture is one such example [178]. *Reproduced with permission from Leavitt T, Hu MS, Marshall CD, Barnes LA, Lorenz HP, Longaker MT. Scarless wound healing: finding the right cells and signals. Cell Tissue Res 2016; 365(3):483–93.*

or specific cell types led to the concept of the totipotent ES cell [179,180]. It was immediately recognized that these cells held the potential to be used for regenerating dysfunctional organs and tissue. Equally obvious were the potential ethical problems related to using cells from human embryos. Whereas other types of stem cells have been used successfully in clinical therapies, such as hematopoietic stem cell transplantation for hematologic diseases and malignancies, therapies involving ES cells remain entirely experimental.

In an early attempt at tissue regeneration using ES cells, Fraidenraich and colleagues injected wild-type ES cells into mice predisposed to death by cardiac failure. The remarkable result of this experiment was that the mice were rescued from their lethal phenotype through a mechanism that appeared to involve signaling factors released by the ES cells [181].

A potential barrier to using therapeutic ES cells in humans is the host immune response against these foreign cells. Immunocompetent mice mount a robust immune response against human ES cells, leading to limited ES cell survival, although this response can be mitigated with the use of immunosuppression [182].

Another difficulty with using ES cells in human therapy is the formation of teratomas; this is unsurprising because the origin of the ES cell's discovery was in the study of teratoma-forming embryonic cells. Undifferentiated ES cells transplanted into ectopic sites, such as the heart, consistently form teratomas [183]. However, advances in the directed differentiation of ES cells toward specific fates should make teratoma formation less of a concern [184].

Improvements in the purifying and characterizing ES cells have allowed for trials testing their efficacy in human diseases. In a phase I/II clinical trial, patients with macular degeneration were treated with retinal pigment epithelium cells derived from human ES cells and with immunosuppression [185]. Several patients experienced improvements in visual acuity up to 12 months after treatment, a result that would not be expected in the absence of treatment.

Human oligodendrocyte precursor cells derived from human ES cells showed promise in treating spinal cord injury in preliminary rodent experiments [186]. However, human spinal cord injury patients treated with similar cells in a phase I trial showed no improvement after treatment [187]. These results highlight that promising results in animal models are not easily translated to human applications, and that novel stem cell therapies must be tested rigorously in human trials. In addition to macular degeneration and spinal cord injury, diabetes, myocardial infarction, and Parkinson disease are among human diseases that are the subjects of ongoing clinical trials involving ES cells [187].

Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are nonhematopoietic bone marrow stromal cells that were initially isolated based on their ability to adhere to plastic culture plates. These cells are unique in that they differentiate into

mesenchymal lineages such as cartilage, fat, muscle, and bone [188]. MSCs are a heterogeneous group of cells that have had populations isolated not only from the bone marrow but also from adipose tissue and amniotic fluid. Based on their ability to expand in vivo and differentiate into multiple mesenchymal tissue types, these cells are thought to be an ideal source of autologous stem cells used for promoting wound healing and/or scar-reducing therapies [189]. MSC therapy avoids rejection and the ethical and moral concerns associated with ES cell therapies.

MSCs may affect wound healing and tissue regeneration through many different avenues. Once transplanted, these cells migrate to the site of injury or inflammation, where they may stimulate the proliferation and differentiation of resident progenitor cells, secrete growth factors, participate in remodeling [188,190], and modulate the immune and inflammatory responses in the wound bed [191].

A wealth of clinical data attest to the safety of bone marrow–derived MSCs, and emerging data support adipose-derived mesenchymal cells as possessing a similar safety profile to bone marrow–derived MSCs [192,193]. Therefore, MSCs could be used to affect various pathways involved in wound healing, including inflammation, aging, and cellular senescence.

Several examples of human wound healing investigations exist using MSCs. The first was a small trial using a fibrin glue vehicle in both acute and chronic wounds. This study demonstrated that topical application of autologous passage 2–10 bone marrow–derived MSCs, combined with fibrin spray, allowed acute surgical wounds and chronic lower extremity ulcers to heal faster. The wound healing speed increased in a manner directly proportional to the number of cells applied [194].

A larger study evaluated patients with various nonhealing wounds. Bone marrow–derived MSCs were applied with a dermal scaffold to wounds, with or without autologous skin grafts. Results showed accelerated healing in wounds treated with MSCs [195]. A limitation of this study was that the cells were not passaged and flow cytometry was not used for isolation. MSCs are known to represent only 0.001% of nucleated cells in the bone marrow; therefore, the cell population used in these experiments was likely heterogeneous and may have contained tissue macrophages that would also assist in wound healing [188].

In a randomized controlled trial in which patients with critical limb ischemia received injections of either allogeneic circulating MSCs or control solution, no difference in outcomes was seen [196]. In a later randomized trial, 24 patients with nonhealing ulcers were randomized to receive autologous cultured MSCs or control treatment [197]. Those treated with MSCs experienced greater symptom improvement and ulcer healing compared with the control group.

Several small, nonrandomized human pilot studies suggested improved healing rates in patients with limb ischemia after treatment with bone marrow–derived mononuclear cells [198–205]. Designed to expand on these results, the JUVENTAS trial is perhaps the most prominent clinical investigation to date based on bone marrow–derived cell therapy [206]. This trial included 160 patients with critical limb ischemia thought to be nonrevascularizable. Subjects received either injections of bone marrow–derived MSCs or placebo injections. There was no significant difference between groups in rates of amputations or quality of life measures. These results confirm the importance of verifying promising preliminary results with well-designed, randomized trials.

Adipose-derived stromal cells (ASCs) are a potentially promising source for therapeutic cells because they are easily isolated from a patient's own fat tissue and exhibit osteogenic and adipogenic activity in vivo and in vitro [207]. Data from animal models indicate that autologous and allogeneic ASCs can regenerate tissue after injury [208]. Two noncontrolled human trials with small numbers of patients demonstrated improved wound healing after the administration of ASCs [209,210]. However, these results must be interpreted with caution because of the absence of control groups. In a small randomized human trial, a product containing ASCs and fibrin glue accelerated closure of perianal fistulae, which share similarities with nonhealing skin wounds [211]. However, a larger follow-up study showed no improvement with ASC administration compared with control treatment [212].

Further research is needed to characterize MSCs and their niches. As purification and enrichment techniques improve, the role of MSCs in wound healing will gain clarity. Defining the direct role of MSCs in wound repair, as well as their effects on other cells, will guide their future therapeutic potential.

Epidermal Stem Cells

As mentioned previously, the epidermis in humans is a dynamic structure undergoing constant renewal. Epidermal turnover is estimated to take place over 60 days in humans, a process that requires a continuous supply of differentiated cells. Epidermal stem cells have a high capacity for self-renewal, as evidenced by the number of daughter cells that undergo terminal differentiation into keratinocytes [213]. Several stem cell niches are present in the epidermis. The best-characterized are the interfollicular epidermal stem cells and the hair follicle bulge region.

The interfollicular epidermis (IFE) is the region of epidermis located between hair follicles. Under normal homeostatic conditions, IFE stem cells defined by expression of the gene *Lrig1* of the basal layer of the epidermis divide at a steady rate to provide new keratinocytes to populate the epidermis [191,214]. These cells may also contribute to the growth of hair follicles and sebaceous glands [214] Whereas the IFE can receive contributions of cells from other structures, such as the hair follicle, it is also capable of repairing and renewing itself after injury in the absence of these other cells [191].

A separate population of epidermal stem cells defined by embryonic expression of *Lgr6* represents a primitive stem cell population that establishes all lineages of the skin, including cells of the hair follicle, sebaceous gland, and interfollicular dermis. In postnatal life, *Lgr6*-expressing cells reside above the hair follicle bulge and contribute to maintaining the sebaceous gland and the IFE [215].

The hair follicle is a complex structure with several distinct regions whose cellular composition has been exceptionally well-studied. Each regions appears to contain a unique population of hair follicle stem cells (HFSCs). It is clear that HFSCs can repair the hair follicle itself after injury. However, an unresolved question is to what extent HFSCs are critical for regenerating the injured epidermis.

The first hair follicle stem cells to be discovered were those residing in the hair follicle bulge region. These cells are characterized by expression of the genes *Krt15*, *Lgr5*, and *Gli1* [191,216]. Initial experiments showing that these cells are present in the epidermis after a scratch injury suggested that they may participate in epidermal repair [217]. However, subsequent experiments showed that the presence of bulge cells in the epidermis is short-lived. It is likely that the contribution of these cells to long-term skin repair is minimal, and that their main role is to regenerate the hair follicle [191].

The junctional zone of the hair follicle is located above the bulge and adjacent to the sebaceous gland. It contains a complex population of stem cells that generally express *Lrig1* but otherwise have different gene expression profiles and roles in regeneration [191,214]. Those that express *Lgr6* in the embryo form the hair follicle, sebaceous gland, and interfollicular dermis. Postnatally, these cells contribute to repair of IFE and hair follicles. Because these *Lgr6*-positive cells are capable of forming several skin structures, they may represent a primitive skin stem cell [215].

Given the important role of epidermal stem cells in skin formation and regeneration after injury, the possibility of using them in a therapeutic role after injury is intriguing [216,218]. Preliminary experiments in animal models have yielded some promising results, such as epidermal stem cells transplanted onto rat wounds [216,218]. However, a major limiting factor in the therapeutic use of epidermal stem cells is their scarce availability and the difficulty in obtaining them. In patients most in need of new keratinocytes, burn victims, there may not be enough autologous cells left in certain situations to make this a viable clinical tool. At this point, the therapeutic potential for epidermal stem cells is largely theoretical, but research continues to develop at a rapid pace [219].

Induced Pluripotent Stem Cells

Difficulties inherent in deriving and using human ES cells led to interest in generating pluripotent cells from other sources. In a landmark paper, Takahashi and colleagues described the transformation of adult dermal fibroblasts into induced pluripotent (iPS) cells using a specific combination of transcription factors [220]. Generation of iPS cells has been achieved from other human cell types, such as keratinocytes [221]. Using similar processes, fibroblasts may be transformed directly into functional, differentiated cell types such as neurons and cardiac myocytes [222,223]. The potential to derive iPS cells from adult tissue avoids the logistical and ethical problems associated with ES cells. Theoretically, iPS cells may also be used in an autogeneic fashion, avoiding the issue of immune rejection.

Translating the potential of iPS cells into human therapies has been challenging. In the only human trial to date involving the therapeutic use of iPS cells, sheets of retinal pigment epithelium cells derived from iPS cells were implanted into the retina of a patient with macular degeneration. Although the patient reportedly experienced improved vision, the trial was halted owing to concerns about mutations detected in the iPS cells [224].

Advances in molecular biology have the potential to refine and improve methods for generating therapeutic iPS cells. For example, the ability to edit specific genetic sequences using clustered regularly interspaced short palindromic repeats—Cas nuclease 9 may allow for the generation of iPS cells with specific traits that could increase their utility in specific disease processes [225]. In addition, the ability to generate functional organoids from iPS cells may prove to be useful for replacing dysfunctional organs in humans. Various groups have used iPS cells to generate organoids resembling cerebral cortex [226], intestine [227], and kidney [228], among others [229] (Fig. 5.7).

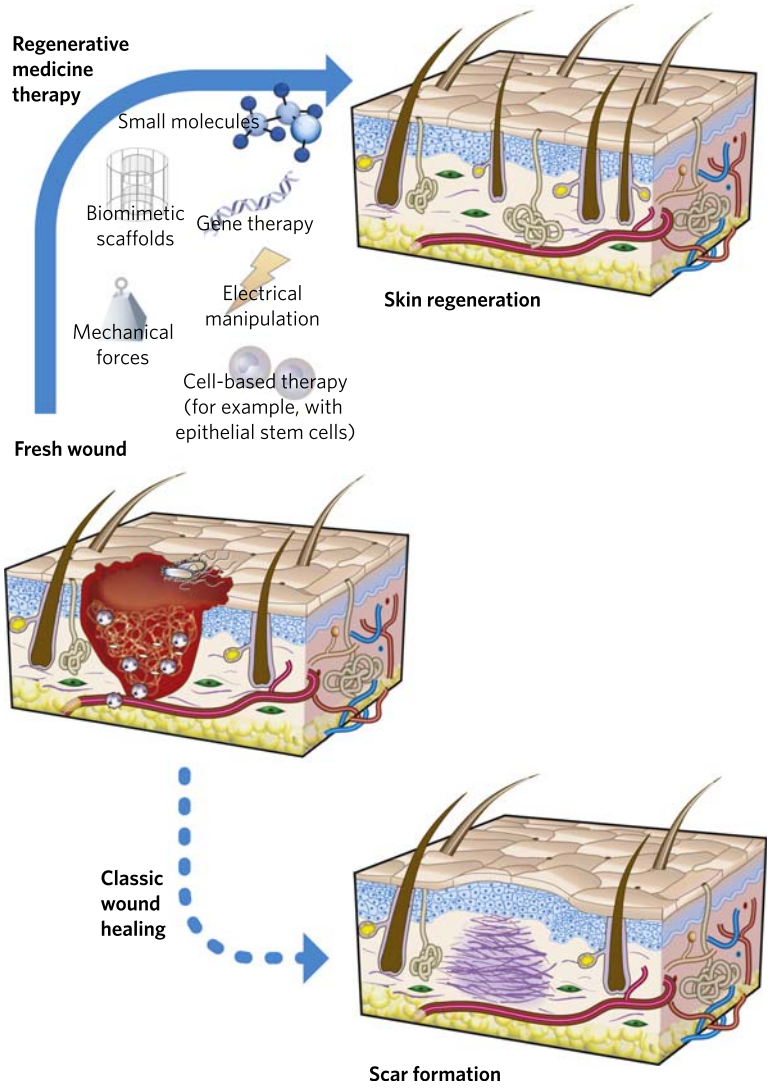

FIGURE 5.7 **Potential therapies for reducing scar formation during wound repair.** To manipulate wound repair to become more regenerative than scar forming, strategies include the use of biomimetic scaffolds, manipulation of the mechanical environment (for example, negative-pressure wound therapy to increase healing) or the electrical environment, the administration of small molecules, the use of gene therapy approaches, and the use of cell-based strategies (including administration of epithelial stem cells). All of these elements have been demonstrated to have an effect on in vitro and in vivo models of wound healing as single-agent therapies. In theory, many of these elements could be combined to recreate a receptive environment (or "soil") to promote regeneration. Combining these with the appropriate stem cells (or "seed") will undoubtedly alter the result of wound healing in humans. *Reproduced with permission from Gurtner GC, Werner S, Barrandon Y, Longaker MT. Wound repair and regeneration. Nature 2008;453(7193):314–21.*

PERSPECTIVE

Our understanding of adult stem cell populations, the complexity of embryonic development, and the redundancy of the adult skin wound healing mechanism has revealed that targeting a single-cell signaling cascade will not be sufficient to recreate scarless wound healing in adult mammals. Rather than the depth of our understanding leading to radical innovations, research has revealed a complex web of interactions among tissue types, structural proteins, developmental and highly evolutionarily conserved growth factors, external forces, and cell-to-cell interactions. On an optimistic note, the number of products for improved wound healing, both acute and chronic, has exploded, giving patients new options and hopes for improved outcomes and wound closure. Unfortunately, however, skin regeneration remains elusive.

With the US population aging and accumulating comorbidities, new solutions are needed that combine scar treatment strategies to prevent the cost of wound healing complications from increasing. Perhaps most exciting, our

understanding of stem cell niches and engraftment issues has improved to the point where autologous and allogenic stem cell therapy may become a therapeutic reality. If scarless wound healing cannot be accomplished in healthy adults by activating appropriate "self" signals, it is possible that engineered and cultured grafts may provide the necessary leap to accomplish regenerative healing.

List of Abbreviations

5FU 5 fluorouracil
ASC Adipose-derived stromal cells
CTGF Connective tissue growth factor
Cx Connexin
E Embryonic day
ECM Extracellular matrix
ES Cell embryonic stem cell
FGF Fibroblast growth factor
HFSC Hair follicle stem cell
IFE Interfollicular epidermis
IL inTerleukin
iPS Induced pluripotent stem
MMP Mixed metalloproteinase
MSC Mesenchymal stem cell
PDGF Platelet-derived growth factor
Smad3 Mothers against decapentaplegic homolog 3
TGF-β Transforming growth factor β
VEGF Vascular endothelial growth factor
Wnt Wingless type

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CHAPTER

6

Progenitor and Stem Cell Heterogeneity: Using Big Data to Divide and Conquer

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INTRODUCTION

Traditional analysis of cells has relied on pooling of RNA or protein from hundreds of thousands of cells and displaying aggregate or average expression of replicate samples. These tools, which include polymerase chain reaction (PCR), microarrays, and western blotting, have been powerful in unfolding transcriptional networks, signaling cascades, and metabolic pathways, to advance our knowledge of disease and therapy. However, the underlying assumption with these techniques is that the population average represents the dominant biological state within the population. This assumption is flawed in most cases because the activity of each cell of the population is not reflected by the population average. Importantly, population averages are unable to capture the activity of rare but critical cells such as stem cells, or transiently amplifying cells such as progenitor cells (Fig. 6.1A). Interrogation of these rare but critical cells could prove to be key regulators of disease progression and therapy.

With the evolution of single-cell technologies such as high-throughput sequencing (HTS), it is possible to evaluate single cells with a high degree of comprehensiveness. These technologies provide information about transcript expression, gene fusions, mutations, or single-nucleotide polymorphisms in individual cells. Outlier cells are no longer considered errors in measurement by default, but can be tested for the presence of a unique function. It is also possible to detect whether a cell population is homogeneous or if cells display heterogeneity by clustering the single-cell data into subpopulations of cells that exist in metastates. These subpopulations can then be tested for functional relevance in tissue homeostasis, repair, and disease (Fig. 6.1B).

In homeostasis, cellular subpopulations function in a stable yet adaptable population equilibrium [1,2]. For example, in the skin, epithelial cells that display a high turnover rate are maintained by distinct subpopulations of self-renewing epithelial stem cells [3]. Similarly, in the bone marrow, the transcriptional, epigenetic, and functional heterogeneity of hematopoietic stem cells determines their cell cycle potential and differentiation ability [4,5]. A second level of complexity arises in tissue injury and repair. After tissue injury, various cell types need to be activated in spatiotemporal concert to bring about healing. In wound repair, for example, multiple cell types within the epidermis, dermis, hypodermis, and circulation must coordinate to bring about healing [6]. Single-cell technologies have revealed heterogeneity within several of the cell types involved in this repair process [7]. However, in tissues such as the heart and the brain, resident cells are still largely considered homogeneous owing to limited access to these tissues in the normal human state.

Cellular heterogeneity also has a critical role in disease and consequences for how the diseased tissue is diagnosed and treated. In cancer biology, tumors display intertumor heterogeneity in which genetic and phenotypic variations are observed between individuals with the same type of tumor, or between tumors in different tissue types of the same individual. However, tumors also exhibit intratumor heterogeneity in which cells within the same tumor exhibit differences in gene expression, cellular morphology, motility, proliferation, metabolism, metastatic potential, and recurrence potential. Alterations in these cellular characteristics can be determined using next-generation HTS

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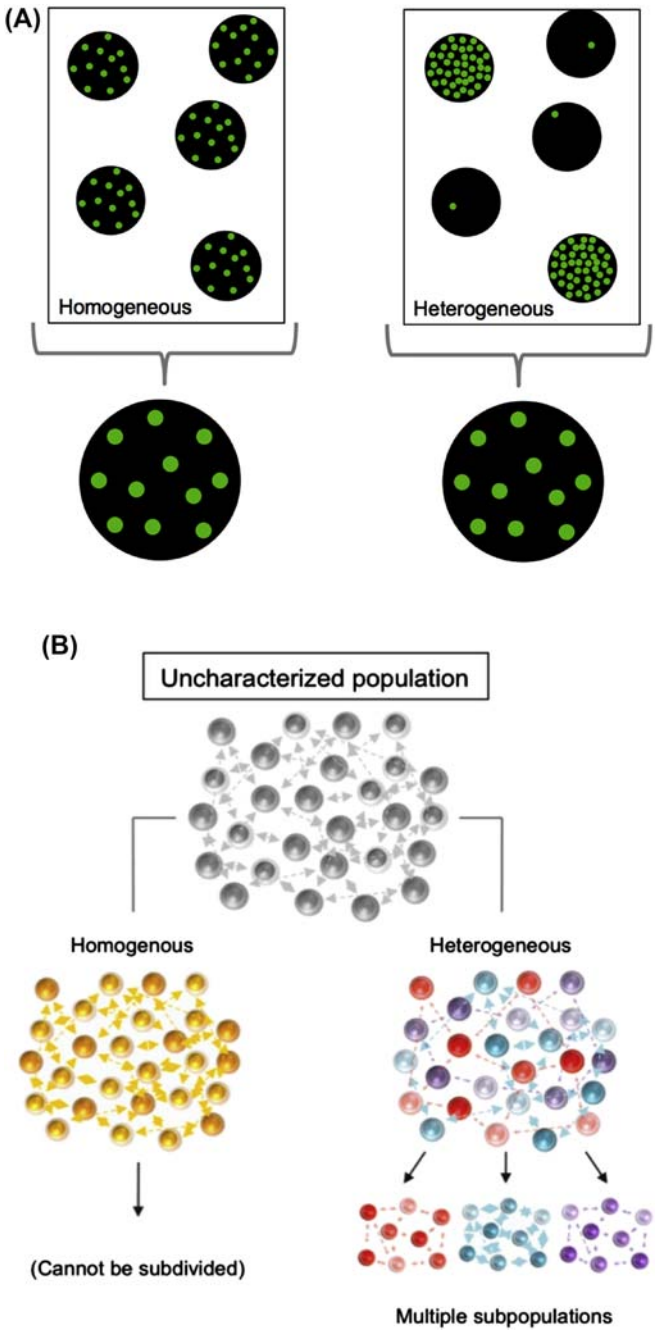

FIGURE 6.1 **Population assays versus single-cell assays.** In population-averaged techniques such as traditional microarray analysis, messenger RNA (*green*) from all cells (*black circles*) are pooled together and the aggregate expression level is reported. When studying heterogeneous populations, such as in cancer and stem cell biology, this approach can lead to considerable loss of information (A). Using single-cell analysis, it is possible to determine whether the population of cells can be further subdivided into subpopulations that are distinct from each other (B).

aided by computational analysis. These technologies have also made it feasible to detect mutational burden and the temporal order of mutations within tumors [8].

Traditional population assays cannot detect cellular heterogeneity because they obscure the response of individual cells and cellular subpopulations. Population assays also require hundreds of thousands of cells to be analyzed, which makes it difficult to study cellular heterogeneity in relatively small samples that are hard to obtain. In comparison, single-cell technologies can determine accurately and comprehensively differences at the genomic,

transcriptional, translation, or epigenetic level. These technologies can assay rare cells. In addition, they allow for analysis of cells in an unbiased manner without the use of markers a priori.

Although single-cell technologies are associated with several advantages, it is necessary to determine the need for resolution at the single-cell level. This is critical considering that the human body is composed of approximately 37.2 trillion cells [9] and each cell generates large datasets of information that must be analyzed computationally and mathematically. Once the need for single-cell analysis is established, it is essential to understand whether the biologic question requires a broad net to be cast to capture information about cellular behavior, or whether a targeted approach is required to reveal only certain cellular features with accuracy and precision. Thus, accuracy, precision, and comprehensiveness determine which technique is selected to answer the biologic question. Accuracy is the measure of certainty or validity that the measured value is close to the true value [10]. It is estimated by comparing the measurements generated by the single-cell technique with a reference standard technique (such as quantitative PCR for transcriptomic analysis). Precision is the ability to replicate or reproduce a measurement. In transcriptomics, high precision is associated with a narrow distribution of gene expression values [10]. Comprehensiveness or sensitivity is the amount of information obtained per cell. In transcriptomics, comprehensiveness refers to the total number of genes that can be detected [10,11]. Advanced single-cell technologies such as RNA sequencing (RNA seq) can detect more than 5000 genes per cell [12]. In addition to these three criteria, considerations such as cost-efficiency, sample size effects, and false discovery are also important for deliberation when comparing and developing HTS techniques [11,13].

This chapter describes the rapidly evolving single-cell technologies that have been used to study cellular heterogeneity. It explains how cellular subpopulations are discovered without prior bias. Finally, it addresses the impact of single-cell technologies on biology, regenerative medicine, and cellular therapy.

SINGLE-CELL ISOLATION

To study heterogeneity and gather data on a single-cell level, cells need to be isolated with accuracy. Most genomic and transcriptional analyses assay nuclear content from individual cells and do not require the isolated cells to be alive. On the other hand, proteomic and metabolic measurements require live cells, which makes single-cell isolation a more challenging process. The classic method of isolating single cells involves manual pipetting followed by serial dilutions. Manual pipetting works only on microliter volumes, is cumbersome, and is difficult to scale. This has led to the use of robots such as the Mosquito HTS, which ensures repeatable nanoliter pipetting irrespective of the viscosity of the cell suspension or environmental conditions [14]. Automated liquid handlers can also scale experiments with ease, converting a 96-well assay into a 1536-well format [14].

In situations in which cells need to be isolated directly from a tissue sample or a surface, laser-capture microdissection can be used [15]. This technique requires a trained histologist to isolate the cells of interest accurately based on morphological properties or changes in fluorescence. To increase the speed of this technique, an automated laser microdissection technique called laser-enabled analysis and processing has been developed [16]. However, laser microdissection can be cumbersome, it may rupture surrounding cells, and it may isolate individual cells incompletely. For these reasons, this technique is not frequently used to obtain cells for HTS.

For decades, fluorescence-activated cell sorting (FACS) has been the default technique to sort cells into well-defined populations. It has become the most widely used technique to isolate single cells for high-throughput studies. FACS can rapidly sort single cells at a high level of purity into 96- or 384-well plates at 10,000 cells per second, a pace that is impossible to match manually [17]. FACS systems work by passing cell suspensions through a small nozzle (70-100 μm in diameter) that creates a continuous stream of droplets, each droplet of which contains a single cell. Electrically charged plates are then used to deflect the droplets containing cells of interest into a microwell plate based on the physical, chemical, or optical properties of the cells that are enhanced using antibodies conjugated to fluorescent probes [15]. Sorting single cells through FACS requires a priori knowledge of surface markers for specific cells and can be difficult to perform in scenarios in which there are limited cells, such as early-stage reproduction and characterization of stem cells (Fig. 6.2). Moreover, the high flow rate and pressure can potentially damage delicate or large cells such as adipocytes as they move through the machine [18]. Most important, FACS does not allow for tracking an individual cell over time, which makes it difficult to determine the dynamic behavior of a cell.

Microfabrication, precision engineering, and rapid prototyping techniques have led to the development of microfluidic devices that allow for sorting low cell volumes on miniaturized devices with rapid throughput [19]. These devices contain thousands of integrated fluid channels, valve control surfaces, and reservoir chambers to separate individual cells [2]. In addition, they reduce the size of the sorting equipment and eliminate biohazardous aerosols

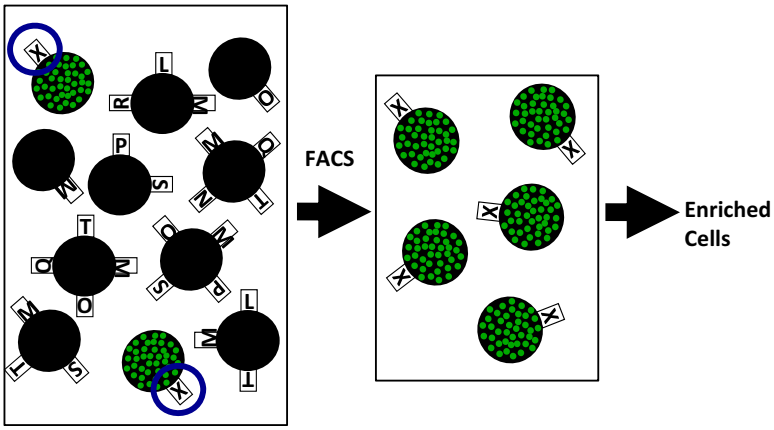

FIGURE 6.2 **Enriching single cells using fluorescence-activated cell sorting (FACS).** FACS can reliably test up to 15 proteins allowing for determination of signaling pathways and enrichment of cells based on surface markers. However, surface markers for enrichment are selected a priori and it is important to determine the best surface marker that can reliably enrich cells for therapeutic use.

[20]. Microfluidic technologies can be either active or passive. Active systems use external fields such as acoustics, electric, magnetic, or optical to displace individual cells into microchambers. The commercially available dielectrophoresis array, for example, uses a nonuniform electric field to exert forces on cells suspended in a liquid and trap them into "cages." This allows for the separation of cells of interest from complex, heterogeneous samples. Passive systems, on the other hand, use inertial forces, filters, and adhesion mechanisms to sort cells [18,20,21]. The commercially available C1 chip from Fluidigm Corporation (South San Francisco, CA) is an example of a passive system in which hydrodynamic traps in a microfluidic channel allow for the isolation of a single cell from a heterogeneous sample. The cell is then subjected to RNA isolation and complementary DNA (cDNA) synthesis within the trap. This system can be scaled to accommodate many traps (up to 800 for the C1 chips), but the largest disadvantage is that the cell pool must be homogeneously sized because the microfluidic traps are sized for an average cell. If the cells are too large, they will rupture before reaching the trap. If the cells are too small, the traps will capture doublets or multiplets [22].

Droplet microfluidics is an emerging field of microfluidic technology in which active or passive systems are used to capture cells within discrete micrometer-sized aqueous (micro)droplets. The flow rate of the aqueous fluid determines the number of droplets that are unoccupied by cells. Each occupied droplet acts as a miniaturized reaction vessel and allows thousands of single cells to be analyzed in parallel every second. Whereas the droplets allow for fluorescent imaging and PCR analysis, they can also keep the cells live for a few hours to enable the analysis of dynamic cellular behavior such as enzyme kinetics or the response to drugs, antibiotics, biologic, and environmental factors. The commercially available 10X platform for example, uses droplet technology for single-cell genome sequencing and RNA seq [23]. In the RNA seq platform, cells are run through a microfluidic chip and single cells are individually encapsulated within gel beads. Each bead has a unique bar code to identify the source cells. The cells then undergo reverse transcription to create bar-coded cDNA. All cDNA from a single cell share the same bar code. The cDNA is then fragmented, amplified, and sequenced. Full sequences are reconstructed from the short-read sequences and expression levels for single cells are determined based on the number of transcripts expressed with the same unique bar code. This technology has been employed to investigate chimerism in immune cell populations, screening for Clustered Regularly Interspaced Short Palindromic Repeats interference, and testing single nucleotide polymorphisms in noninvasive prenatal testing, among other applications [23–25].

ACQUIRING SINGLE-CELL DATA

Once individual cells are collected, single-cell analysis can be performed. The flow of biological information in cells is from DNA to RNA to protein, with DNA containing the genetic information of all cellular organisms, with RNA functioning as the messenger of this information and proteins acting as the working force to determine the phenotype of the organism. Random mutations bring about changes to the DNA. Injury and disease bring about changes in the transcribed RNA and protein. Sometimes there are modifications in the phenotype with no changes to the DNA sequence owing to changes in gene expression. These alterations, called epigenetic changes, can be

influenced by various conditions including age, the cellular microenvironment, or disease states. This section describes the various high-throughput techniques developed to study changes in the DNA, RNA, protein, or epigenetics of individual cells.

Single-Cell Genomics

Next-generation DNA sequencing allows for the comprehensive study of minute amounts of DNA from an individual cell. This technology has uncovered the evolutionary history of cells, genomes of unculturable microorganisms, and genetic mosaicism in normal physiology, disease, and cancer [26,27]. Genetic mosaicism is the presence of two or more populations of cells with different genotypes within the same individual, developed from a single fertilized egg. Although it has long been known that cancer is a mosaic disorder, in somatic tissues, early studies of genetic mosaicism were limited to abnormalities in skin development such as epidermolysis bullosa and ichthyosis. However, the advent of single-cell DNA sequencing unearthed variations in the chromosome, copy number, and single nucleotide sequences in a variety of tissue types, and mosaicism is now seen in a diverse range of clinical disorders that can be gonadal, somatic, or gonosomal [28].

To obtain single-cell genomic data, single cells are isolated and subjected to whole-genome amplification. This can be achieved through either PCR amplification or isothermal methods such as multiple displacement amplification [18]. The isothermal methods have greater coverage or comprehensiveness compared with the PCR-based methods; however, they display lack of uniformity or accuracy and precision [26]. Therefore, most single-cell genomic approaches use a hybrid method that consists of a limited isothermal amplification step followed by PCR amplification. These hybrid techniques include displacement degenerate oligonucleotide-primed (DOP)-PCR and multiple annealing and looping-based amplification cycles (MALBAC) and differ based on whether degenerate primers or random primers are used for amplification. DOP-PCR and MALBAC have uniform coverage that results in greater sensitivity and accuracy [29,30].

Once the genome is amplified, it is subjected to single-cell exome sequencing or entire-genome sequencing [31]. An important consideration while sequencing is that false variants increase as the size of the genome region increases. Furthermore, errors can be introduced in any stage, including the single-cell isolation and whole-genome amplification steps. Hence, it is imperative to develop tools that differentiate technical aberrancies and noise. The quality metrics include visual conformation of isolated cells as well as quantification of the whole-genome amplification product.

Single-Cell Transcriptomics

Whereas genomics allows for the identification of genetic alterations within cells, transcriptomics allows the investigator to understand changes in the function of cells. Single-cell transcriptional studies emerged with the integration of single-cell quantitative polymerase chain reaction (qPCR) into microfluidic platforms, allowing for massively parallel qPCR reactions on a small chip [32]. The Biomark chip from Fluidigm Corporation, for example, provides a highly sensitive platform that allows for probing of individual cells for the expression of 96 select genes, providing a readout in less than 24 h after sample collection. This platform enables cDNA volumes to be detected that are 1000 times less than those required for traditional qPCR reactions. In this method, a single cell is sorted by FACS into a well of a 96-well plate. The cDNA conversion step is combined with a low-cycle reverse-transcriptase (RT)-PCR step that preamplifies the cDNA with the select 96 primers before it is loaded onto a microfluidics chip. The microfluidics chip is subjected to qPCR in a Biomark machine (Fluidigm Corporation) where the cDNA from each cell is amplified by each of the 96 primers, which results in 9216 data points (Fig. 6.3A). This microfluidic-based qPCR technology generates highly accurate and precise gene expression data and has been used to determine heterogeneity among a wide range of cell types including fibroblasts, adipose stromal cells, glioblastomas, and hematopoietic stem cells [2,33–36]. The main downside of this method is that the number of genes that can be evaluated depends on the size of the microfluidics chip [37].

HTS of whole transcriptomes through RNA seq enables profiling of the transcriptome. This technology enables the detection of absolute levels of gene expression, gene fusions, single nucleotide variants, insertions, deletions, and weakly expressed genes. Unlike hybridization methods such as microarrays, RNA seq does not require prior knowledge about the organism (species or transcript-specific probes), which enables the identification of both known and novel transcripts. It also provides a signal-to-noise advantage over microarrays by eliminating cross-hybridization, nonideal hybridization, and DNA contaminants. DNA contaminants are negated by unambiguously mapping DNA

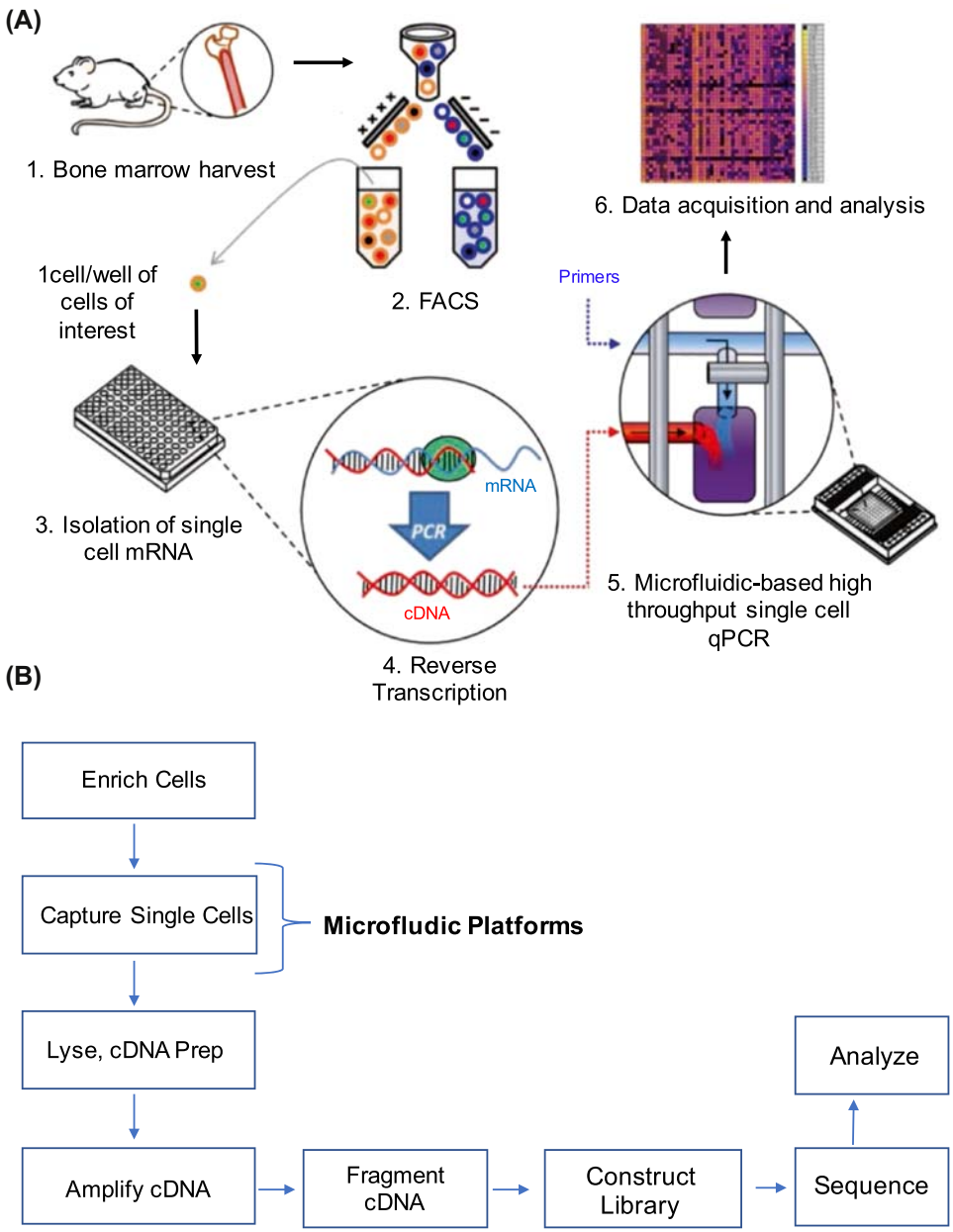

FIGURE 6.3 **Single-cell transcriptomics.** Schematic of high-throughput, microfluidic chip-based, single-cell transcriptional analysis demonstrates how a single cell is sorted by fluorescence-activated cell sorting (FACS) into each well of a 96-well plate that has been preloaded with reverse transcriptase–polymerase chain reaction (RT-PCR) reagents. A low-cycle RT-PCR preamplification step creates complementary DNA (cDNA) for each individual cell. Single-cell cDNA is then loaded onto the microfluidics chip along with the primer-probe sets for 96 gene targets and quantitative polymerase chain reaction is performed in the Biomark machine, leading to 9216 data points per chip (A). Schematic of single-cell RNA sequencing demonstrates that cells first need to be isolated and enriched by techniques such as FACS. Microfluidic technologies are then used to isolate single cells. Reverse transcription of messenger RNA is performed to produce cDNA that is amplified through PCR. The amplified cDNA is sheared to reduce the length of the sequences and the fragments are sequenced to the number of desired reads. Finally, the sequences are reconstructed and matched to a known library and the copy numbers of transcripts are determined (B).

sequences to unique regions of the genome. Based on its sensitivity and range of expression, there has been an overwhelming interest in using RNA seq on single cells to determine heterogeneity within cell populations, identify rare cells, and characterize poorly defined cells.

Although individual protocols vary slightly, the overall processing of single-cell RNA seq is similar. First, template switching by reverse transcription of messenger RNA (mRNA) is performed to produce cDNA that is amplified through PCR. The amplified cDNA is sheared to reduce the length of the sequences and the fragments are

sequenced to the number of desired reads. Subsequently, the sequences are reconstructed and matched to a known library and the copy numbers are determined [37]. This method allows sequencing of all mRNA molecules, resulting in an unbiased approach to evaluating the transcriptome and allowing for the discovery of novel transcripts or splice variants (Fig. 6.3B) [38–40].

Despite the comprehensiveness of the technique, single-cell RNA seq is associated with a myriad of technical issues that result in uncertainty of the generated data. Most microfluidic platforms require several thousands of cells to be loaded in highly concentrated solutions as starting material before the cells are distributed into individual traps or microdroplets. In most cases, these cells are freshly sorted by FACS to obtain a homogeneously sized population. Although tested for viability before loading into the microfluidic chamber, it is unlikely that all the FACS-sorted cells are live at the start of the assay. In addition, microfluidic separation of cells may cause cell rupturing or the microchambers may capture doublets or multiplets. Several technologies do not allow for the visualization of entrapped cells within the traps or microdroplets before cDNA synthesis. If doublets are captured, it is difficult to distinguish the data obtained from a single cell. Similarly, if the cells are ruptured and only part of the RNA becomes reverse transcribed, it is difficult to separate these data from those of an intact single cell. Many sequencing technologies use spike-in RNA controls (either external RNA controls consortium or custom) to determine the quality and success of the library constructed. These spike-ins provide transcripts at a known sequence, length, and concentration and serve as a quality control for the isolated single-cell RNA. Although used as controls, in several cases, the spike-ins compete and become reverse transcribed instead of the cellular mRNA. mRNA by itself is a delicate molecule and easily prone to degradation. Therefore, strict quality control measures of fragment range in the Bioanalyzer or Experion is helpful.

Single-Cell Proteomics

Proteomics provides information about the biochemical activation state of the translated protein. The activation of a protein is represented by its phosphorylation, acetylation, proteolytic cleavage, ubiquitylation, change in localization, conformation, or abundance within cells [41]. Traditionally, these processes have been analyzed by population assays such as western blotting and immunofluorescence imaging. However, the analysis of these changes in single cells is challenging because of the paucity of protein amplification techniques [18]. FACS has been the most well-established technique for determining the relative expression of surface proteins on single cells; it distinguishes individual cells within a mixed population of cells and enriches live cells based on surface markers [42]. FACS can reliably test up to 15 proteins and enables the determination of signaling pathways and networks within individual cells. However, these are markers selected a priori, and it is important to understand which is the best surface marker that can enrich cells before therapeutic use (Fig. 6.2). When the cells are fixed and permeabilized, FACS also helps in determining the intracellular activation state within individual cells. Most commercial flow cytometers require manual sample preparation of cells; however, improvements have been made in microfluidic platforms that allow for handling, sorting, and flow cytometry [42].

Groups of cells can also be fluorescently bar coded with unique signatures of fluorescent dyes, so that they can be mixed together, stained, and analyzed as a single sample. This results in the possibility of high-throughput FACS analysis. Fluorescent bar coding reduces antibody consumption by 10-fold to 100-fold and minimizes pipetting error, staining variation, and the need for normalization. The robustness of data is increased through the combination of control and treatment samples, and the speed of acquisition of data is enhanced [43].

Mass cytometry is a combination of flow cytometry with mass spectrometry and provides measurement of over 40 parameters simultaneously at a single-cell resolution. This process allows for millions of cells to be assayed, enabling sufficient sampling to identify major cell subsets from the heterogeneous cell sample [44]. Unlike conventional FACS, which uses fluorophores as reporters causing spectral overlap, mass cytometry uses unique, stable, heavy metal isotopes of atomic weights different from those employed in mass spectrometry. This allows for greater number of parameters to be analyzed with little signal overlap between parameters. The instrumentation used for mass cytometry is called cytometry by time-of-flight [44]. Disadvantages of mass cytometry are that it cannot determine forward and side scatter, and hence cannot determine cell size and shape; the acquisition rate is slower than FACS; and cells must be fixed before analysis. Thus, mass cytometry can be used to analyze subpopulations of cells but cannot be used to enrich subsets of live cells based on these markers.

A long-standing challenge in proteomic analysis is the transformation of high-resolution mass spectrometry as a cell population–averaging tool into a single-cell analyzer. Advancements have enabled the identification of 500–800 nonredundant protein groups from single cells using less than 0.2% of the total protein of the cell as starting material

[45]. However, the main drawback is a low signal-to-noise ratio when considering the low levels of protein within a single cell [42].

Single-Cell Epigenetics

Epigenetic modifications such as DNA methylation or histone modifications are functionally relevant changes to the genome that perturb gene expression without altering the DNA sequence. The best-studied epigenetic modification is DNA methylation, which consists of the addition of a methyl group to cytosine residues (5-methylcytosine). Usually, this alteration is inversely correlated with gene expression levels with implications for tumor biology, disease progression, resistance to standard drug treatments, and relapse.

Attempts have been made to study epigenetics at the single-cell level. Single-cell genome-wide bisulfite sequencing (scBS-seq) has been used to assess the epigenetic heterogeneity of DNA. In this technique, the DNA of a cell is treated with bisulfite, which results in DNA fragmentation and the conversion of unmethylated cytosines to thymine [46]. Complementary strands of the fragmented DNA are synthesized using adaptor sequences and random oligonucleotides. This step is repeated several times to obtain enough tagged DNA and copy numbers of each fragment. A second adaptor is integrated, PCR amplification is performed, and cDNA libraries are generated, which are subjected to sequencing [46]. The number of CpGs obtained from the analyzed data depends on the depth of sequencing.

Furthermore, to understand the complex relationship between DNA methylation and transcription in heterogeneous cell populations, scBS-seq of the genome and RNA seq of the transcriptome have been performed on the same cell. Such analyses have clinical implications in contexts such as in vitro fertilization, in which the number of cells for analysis is limited [47].

Advances in sequencing have cleared the way for many other methods to assess single-cell modifications. These include formaldehyde-assisted isolation of regulatory elements followed by sequencing, chromatin immunoprecipitation sequencing, DNase sequencing, Micrococcal nuclease followed by sequencing, and assay for transposase-accessible chromatin sequencing. The general principle of these technologies is fragmentation of DNA and sequencing of the regions that have been bound by DNA-binding proteins [48]. This has allowed for improved profiling of DNA-binding proteins, histone modifications, and nucleosomes [49].

ANALYZING SINGLE-CELL DATA

As detailed previously, single-cell technologies enable alterations in genes, gene expression profiles, and protein production within single cells to be determined [50]. However, single-cell sequencing technologies generate large amounts of data requiring computational infrastructure and expertise. For example, whole-genome sequencing of 100 individual cells at read lengths of 75 base pairs requires about 15 TB of storage space. Subsequently, the quality of these data needs to be assessed so that variations resulting from noise can be distinguished from true biological variations. The vetted data are then normalized and analyzed to reveal subpopulations of cells or information of clinical relevance. Thus, it is essential from the start to understand whether the biologic question requires a broad net to be cast to capture all information about individual cellular behavior, or whether a targeted approach is beneficial to reveal the most important cellular features affecting human health.

Reducing Noise in Single-Cell Data

Noise in data generated from single-cell technologies can be classified into two major types: technical and biological [10,51]. During sequencing, technical variations can occur as a result of insufficient amounts of isolated RNA/DNA, instability of the minute amounts of isolated RNA, problems in amplification of DNA/RNA, and bias during library preparation [51]. It has been estimated that over 80% of variation in expression patterns in single-cell RNA seq results from technical variations in measurement [51]. Biological noise can be caused by differences in the stages of the cell cycle, the epigenetic status of the cell, and the cellular microenvironment. It can also occur owing to the ineffective isolation of initial cells [26]. Biological confounders account for up to 18% of noise in single-cell RNA seq data and are especially problematic when analyzing rare subpopulations of cells [51].

To build accurate clinical models to guide diagnosis and treatment, it is important to minimize variability and noise in single-cell analysis techniques. This can be achieved by identifying and removing low-quality cells through

two major steps: (1) quality control measures at each experimental step, and (2) quality control during data analysis [50].

Quality control at the experimental level can be performed at various stages by testing for the viability and size of the isolated cells, visually inspecting entrapment of cells within microfluidic chambers, testing the quality of DNA/RNA, analyzing the size of the fragments generated, and quality testing the library preparation to ensure the data from each cell reaches a minimum threshold of usability. An interesting solution to detect doublets or multiplets is to mix cell populations from two different species. For example, when studying cardiomyocyte heterogeneity, cardiomyocytes from murine and human cells can be isolated and processed together. During data analysis, seemingly individual cells displaying mixed cDNA patterns can be discarded. The number of single cells analyzed can also be increased, resulting in a higher detection power [52]. However, this approach increases the amount of data generated when the same depth of sequencing is desired and requires greater computation capacity and resources. Another approach is to use nanoliter volumes of sample preparation to yield fewer false positives in gene expression studies compared with microliter formulations [10]. This method requires the use of automated liquid dispensers and robots.

Quality control at the data analysis level can be performed by excluding cells with minimal numbers of reads or excluding cells with a high number of poor-quality reads. Furthermore, research groups have attempted to create models to remove biological confounding factors. One group succeeded in determining where cells lay within their paths of differentiation by creating a model that adjusted for cell cycle−specific changes in gene expression [53].

Normalizing Single-Cell Data

Once clean data are identified, normalization can be performed to compare data from one cell with data from other cells. Normalization of data can be performed through a variety of techniques and can vary depending on the technology used [54]. This has proven to be challenging for all single-cell analysis methods. For bulk RT-PCR studies, housekeeping genes have been used to normalize gene expression against that of a gene with relatively constant expression across cells. However, at the single-cell level, there is significant variation between these housekeeping genes [55]. This has led to normalization with a combination of housekeeping genes or with genes that are best for the tissue being evaluated [56]. Some researchers even suggest using no normalization owing to these significant variations between cells [57].

As with single-cell RT-PCR analysis, there are also multiple methods for data normalization with RNA sequencing [54]. One method involves median normalization, in which the mean count for all cells of each gene is calculated and a size factor is created by determining the median of the fraction of each sample's count over the mean across all samples. The size factor is then applied to all samples. Another method to normalize the data that accounts for technical artifacts is the use of spike-ins [54]. This involves introducing synthetic transcripts into each cell's library at a known concentration. Knowledge of what the expected and observed counts are can then be used as a multiplicative factor to account for technical errors that occur during the process.

Mathematical Identification of Cellular Subpopulations

Once the data have been vetted, they can be used in various ways. One specific purpose is to find important subpopulations with unique functions [50]. Single-cell technologies have demonstrated that cells that were once thought to be homogeneous display significant variations in their functional profiles and instead exist as subpopulations. However, perfectly equivalent expression profiles between any two cells are highly unlikely, which pushes the biostatistician to determine how best to group cellular subpopulations.

A variety of techniques have been used to cluster cells within a population and reveal subpopulations. Two main categories of clustering include hierarchical and partition clustering. However, many variations exist within these groups [58].

Hierarchical clustering functions by combining or dividing groups and creating a hierarchical structure that demonstrates this order (Fig. 6.4). The two main methods within this technique include agglomerative nesting and divisive analysis [47]. Agglomerative nesting methods involve first arranging the data into a series of sets with one object in each set. For the purposes of single-cell analysis, an object would be considered the data profile from a single cell. A cost function is used to determine which of these sets is "cheapest" to combine. The two objects are then combined, removed from the list, and replaced with a combination of their components. The process is repeated until all items exist within a single group. Divisive analysis differs from agglomerative nesting in that it begins with one set

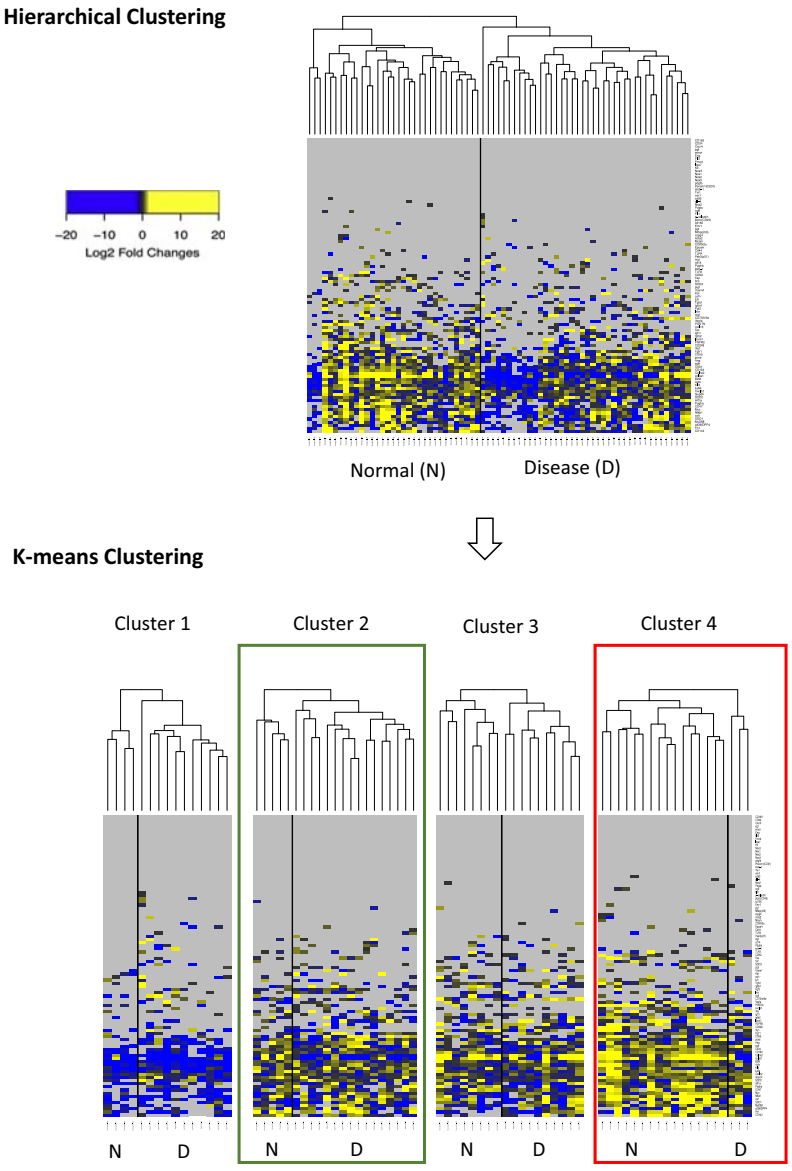

FIGURE 6.4 **Hierarchical clustering followed by k-means clustering can reveal novel subsets that are altered in disease.** Single-cell transcriptional data can be represented by hierarchical clustering with each cell, represented as a column, and each gene, represented as a row in the heat map. There data can further be clustered using k-means clustering to reveal subsets that are altered in disease. Cells in cluster 2 (*green box*) show an increased frequency in the diseased state, whereas cells in cluster 4 show a decreased frequency in the diseased state (*red box*). These cells can then be specifically targeted to test for therapeutic outcomes.

containing all objects. The object with the greatest dissimilarity from the rest of the objects is separated from the group. The remaining objects are then evaluated to see whether they should be included in this separated group. The process is repeated until there are as many clusters as there are objects. Each separation is demonstrated within a dendrogram to show the level of separation among the various groups. Once an object is placed into a group, it cannot be reassigned from that group. The specific mathematical approach to clustering can be varied based on the cost function used within the algorithm.

In contrast to hierarchical clustering, partition clustering involves a set number of clusters that are defined a priori [47]. This method involves placing objects into a cluster with the closest center. The goal is then to minimize dispersion within each cluster through iterative reallocation of the objects within clusters. Unlike hierarchical clustering, this method enables an object's cluster assignment to be changed at any step. Two specific examples of partition clustering are k-means clustering and partitioning around medoids (PAM). The difference between these two methods is that the cluster center in k-means is the average of the objects, whereas in PAM the center is an actual object.

A significant number of modifications have been made to both hierarchical and partition clustering. One example is demonstrated by the addition of fuzzy c-means clustering. The methods of partition clustering listed earlier involve placing an object into a single cluster, which is referred to as "hard assignment" [59]. Fuzzy c-means clustering uses a "soft assignment," which allows placement of an object into multiple clusters. This becomes useful for objects that lie between two clusters.

With partition clustering, a key requirement is to determine how many clusters one should create. Many methods have been presented to attempt to determine the optimal number of clusters, with none obviously superior to the others [59]. These include, but are not limited to, minimum message length criteria with a Gaussian mixture model, minimum description length, Bayes information criterion, Akaike information criterion, and gap statistics. Even with these objective measures, there is no perfect method to determine how many meaningful clusters exist within a given data set.

DETERMINING SUBPOPULATIONS

Although cells are defined by their intracellular characteristics (transcription, translation, epigenetics, metabolism, etc), the only way to isolate live cells is to use markers on their surface. Many of these surface markers defined in the literature may have little to no mechanistic relationship to the intracellular function of the cell, but they are selected by educated guesses. For the first time, single-cell technologies allow the correlation of surface markers to the intracellular cellular machinery to ensure that the best surface markers are selected to determine cellular subpopulations. However, to identify the best signature for the cell, a broad net needs to be cast encompassing all surface markers.

Single-cell RNA seq offers the widest resolution and can inform decision making. However, the large datasets generated by this technology contain high amounts of technical noise, masking, or amplifying cellular heterogeneity and make it difficult to define rare subpopulations accurately and precisely. This is like to finding a needle in a haystack [60]. From a technical perspective, it is unclear whether single-cell RNA seq will ever approach the accuracy and precision of single-cell qPCR. Such unreliability is not acceptable for prospectively isolating and functionally testing cell subpopulations and generating therapeutic products that will be used in humans. To overcome these shortcomings, a single-cell approach was developed and validated that leverages the comprehensiveness of single-cell RNA seq and the accuracy and precision of single-cell qPCR [60].

Specifically, this approach uses information from single-cell RNA seq, metaanalysis of publicly available microarray databases, and peer-reviewed literature to arrive at 96 genes with which to interrogate cells of interest (fibroblasts, hepatocytes, etc.). These genes include cell cycle, transcriptional, and cell-specific genes that appear to be sporadically expressed by the three screening analyses. The 96 genes are then transcriptionally analyzed in individual cells by qPCR on a reliable Fluidigm Biomark microfluidic chip. The single-cell transcriptional data are subjected to hierarchical and k-means clustering to validate expression of the 96 genes and accurately determine systematic variations within the cells. This is the first part of the analysis, which determines whether putatively homogeneous cells contain undiscovered subpopulations.

At this point, if there are subpopulations (i.e., autonomous clusters), it is still not clear whether these subpopulations have any differential function. It is possible that the transcriptional clusters are merely a descriptive curiosity. To determine functional relevance requires isolation of the subpopulations and prospective functional analysis. Only cells with differing functionality are considered "true" autonomous cell subpopulations. To prove functional relevance, the subpopulations need to be isolated with surface markers using existing FACS technology. To do this, single cells are subjected to transcriptional analysis for a second time. Mathematically, the original 96 genes can be winnowed to eight or nine "cluster defining" genes, which leaves 80 or more open channels on each chip. These open channels are used to correlate the cluster defining genes blindly with all known surface markers capable of being used for FACS sorting. This results in a surface marker combination with the highest specificity and sensitivity for each subpopulation (receiver operating characteristic curve closest to 1). The surface marker combination identified is used to isolate the subpopulations of interest accurately and precisely using FACS, and the cells are functionally tested (Fig. 6.5). This technology can be used to speciate any cell type, isolate rare cells, and study cellular alterations in any disease state [33].

This single-cell approach has been used to (1) study cellular heterogeneity in various cell types including fibroblasts, mesenchymal stem cells, hematopoietic stem cells, neural stem cells (NSCs), and glioblastomas [7,33,34,36,61]; (2) determine subpopulation alterations in a wide range of diseases such as diabetes, aging, fibrosis, and cancer [34,35,62,63]; and (3) identify surface markers accurately for the prospective isolation of cells for therapeutic use [33].

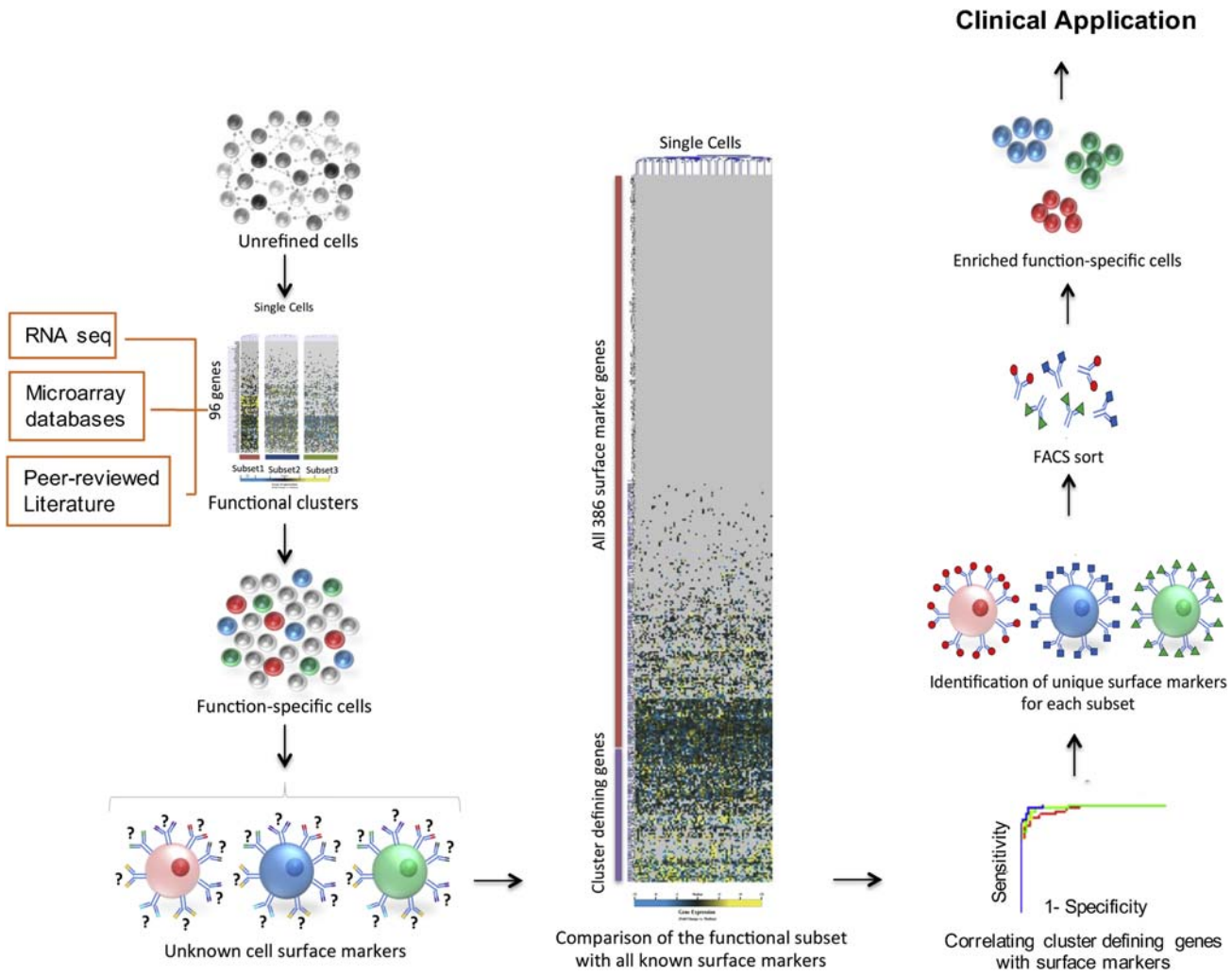

FIGURE 6.5 **Development of a rational framework for the identification and isolation of functional cell subpopulations.** Seemingly homogeneous cells within a population are subjected to single-cell transcriptional analysis to determine cell subsets based on differential gene expression across 96 genes. The 96 genes are selected from RNA sequencing, publicly available microarray databases, and peer-reviewed literature. The subsets are then probed with all 386 surface markers in parallel with the intracellular functional genes to determine the ideal surface markers that select for these clusters. Because the multiplex chip allows for the probing of only 96 genes, at least five chips are used and single-cell transcriptional data across these chips are displayed. Transcriptional data of the 386 surface markers is then blindly correlated with intracellular genes to determine the surface marker combination that identifies the cell with the highest sensitivity and specificity. Finally, antibodies for the selected surface markers are employed to fluorescence-activated cell sort (FACS) and enrich the functional subsets that can readily be used for investigation and therapy.

Development of Cell-Based Therapies

Cell-based therapies have been developed based on 'legacy' surface markers derived from the literature and from historical data. Thus, when trials fail, it becomes difficult to determine the best way in which to proceed. Customized therapies require an in-depth knowledge of both disrupted cellular pathways in diseased tissue and cell surface marker information on cells that can bring about the best therapeutic effect.

The effectiveness of the Needlestack platform was tested to identify and isolate single-cell subpopulations rationally for therapeutic use. Human subcutaneous adipose-derived stromal cells (ASCs) were selected as the cell therapy source [33]. These cells can easily be isolated from the subcutaneous tissue and were tested widely in preclinical trials based on their ability to produce growth factors and deposit extracellular cells. However, ASCs are a heterogeneous group of cells obtained from excluding hematopoietic cells and endothelial cells and enrich CD34-expressing cells from the stromal vascular fraction. If these cells are to be used therapeutically, it is imperative to understand the healing effects of the various cell subsets within this population.

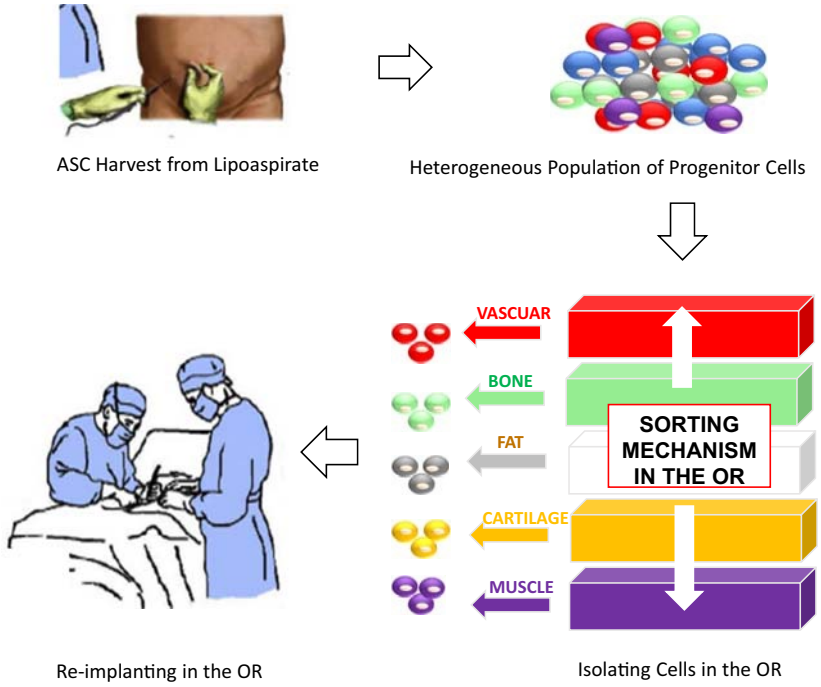

FIGURE 6.6 **Isolating the best cell for any given clinical application.** Single-cell technologies can identify which cells in a heterogeneous population will provide the best outcome for a specific clinical application. Thus, heterogeneous cells such as adipose-derived stromal cell (ASCs) that are obtained from lipoaspirates in the clinic can be enriched for these highly potent cellular subsets in the operating room (OR) and delivered immediately to patients who require therapy.

Hence, ASCs were individually isolated and subjected to single-cell qPCR against 96 genes involved in tissue repair. The gene list was informed from the literature and publicly available microarray databases. The resulting data were subjected to k-means clustering to identify subpopulations. The subpopulation most favorable to wound healing was selected. This subpopulation was defined by 18 genes, which left open 78 channels to interrogate cell surface marker expression in a second set of experiments. Upon combining five chips, it allowed for an unbiased and blinded correlation with 386 surface markers. This blinded correlation (mathematically performed by linear discriminant analysis) identified two surface markers that precisely selected the subpopulation of interest [33].

Next, these two surface markers were used to enrich the ASC subpopulation by FACS, and the cells were delivered to diabetic wounds. Diabetic wounds displayed a normalization of the healing response with the enriched ASCs. Interestingly, the application of ASCs depleted of this important subset demonstrated no effect on diabetic healing, whereas application of the unsorted, heterogeneous ASCs improved but did not normalize diabetic healing. This was the first cell-based targeted therapy to normalize diabetic wound healing in a preclinical setting and could be extended to the treatment of any disease (Fig. 6.6). Importantly, this single-cell method for selecting the best cellular subpopulation exists during a time when US Food and Drug Administration—approved cell-based therapies such as Apligraf and Carticel have shown efficacy and have resulted in reduced overall health care costs for patients.

CLINICAL IMPLICATIONS OF CELLULAR HETEROGENEITY IN TISSUE REPAIR AND DISEASE

Customized therapies need an in-depth analysis of impaired cellular pathways in disease and a granular understanding of cellular subpopulation changes that underlie disease. Although population-averaged assays cannot provide such resolution, the development of novel single-cell technologies provide great promise for targeted basic science and clinical discovery. This section summarizes advancements made using single-cell technologies in understanding the molecular and cellular changes that modulate diabetes, aging, wound healing, cancer, and fibrosis (Fig. 6.7).

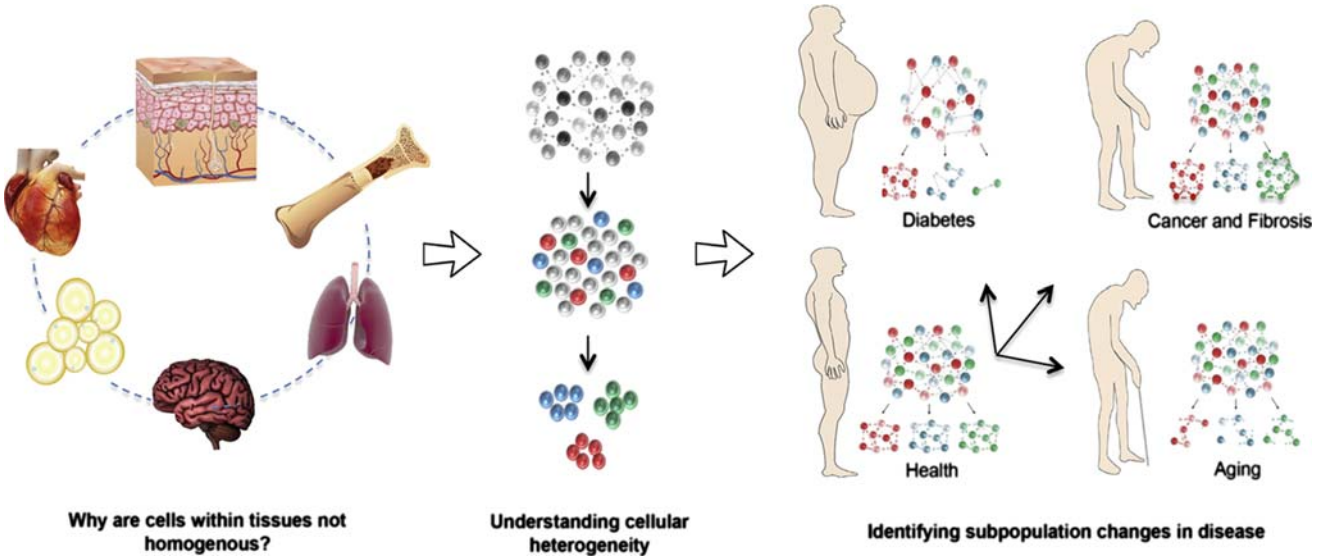

FIGURE 6.7 **Understanding the functional relevance of cellular heterogeneity.** The development of novel single-cell technologies offers great promise for targeted basic science and clinical discovery. These techniques allow for the comprehensive mapping of cells within various tissues in health and inform us about alterations in cellular subsets during aging or diseases such as diabetes, fibrosis, and cancer.

Cellular Heterogeneity in Diabetes

Diabetes brings about cellular and molecular impairments in a wide variety of cell types including stem and progenitor cells leading to tissue dysfunction. In many cases, owing to hyperglycemic memory, these cellular perturbations do not normalize even after a return to normoglycemia, resulting in persistence of tissue dysfunction [64]. Single-cell interrogation of subcutaneous ASCs in type 1 and type 2 diabetes has demonstrated that the global dysfunction in ASCs is brought about by the selective depletion of discrete ASC subpopulations, impairing wound healing in diabetes [33,62]. Similarly, type 1 and type 2 diabetes bring about intrinsic defects within bone marrow progenitor cells through selective depletion in provasculogenic subpopulations. These defects are not correctable by restoring glucose homeostasis [65]. Within the bone microenvironment, single-cell RNA seq has also revealed intrinsic skeletal stem cell impairments caused by hyperglycemic changes within the stem cell niche [66].

Interestingly, single-cell analyses revealed differences within insulin-producing pancreatic β cells, a population long considered to be homogeneous. These studies indicated that adult β-cell subpopulations can differ in size, insulin production, insulin secretion, and precursor cell potential with relevance to an understanding of diabetes and implications for enhancing cell replacement therapies for treating diabetes [67,68].

Cellular Heterogeneity in Wound Healing

Wound repair is an example of a highly heterogeneous tissue with several different cell types working in concert at distinct spatiotemporal stages to bring about healing [6]. Immediately after a wound is formed, neutrophils are recruited from the bone marrow as the first line of defense against bacteria. Classically, neutrophils have been considered a homogeneous population of terminally differentiated cells with a conserved function. Their limited proliferation ability, short life span, and low mRNA content (10–20 times lower than leukocytes) have made it difficult to tease apart the various subsets of neutrophils through population techniques [69]. However, single-cell technologies revealed both phenotypic and functional versatility in neutrophils that extend beyond their antimicrobial activity to their impact on disease and their ability to activate other cells such as macrophages [70–72].

With the evolution of single-cell technologies, the definition of a macrophage has evolved as a cell that engulfs and digests pathogens, particles, and dead cells. It is now accepted that tissue macrophages have the unique ability of plasticity, in which the cells modulate their activation state based on external cues such as the presence of infection, growth factors, and cytokines in their microenvironment [73]. The diversity within macrophages is seen at the phenotypic, genetic, and epigenetic levels, leading to subsets of macrophages that are proinflammatory, antiinflammatory, and provascular, or transitioning between these states. Furthermore, there are macrophages in the adult tissue that originate during embryonic development that are not derived from monocytes [74]. Thus, spatiotemporal

factors within the wounded tissue microenvironment determine the presence of macrophage subpopulations, each potentially with a unique function.

Neovascularization follows the inflammatory phase of repair in every tissue. During this phase, blood vessels are at various levels of maturity. Some vessels are intact and are maintaining blood fluidity, some are leaky and aiding the influx of inflammatory cells, and others are actively undergoing angiogenesis. During angiogenesis, endothelial cells are sprouting and proliferating, whereas pericytes within the basal lamina are activated to scaffold and provide structural integrity to the new vessels. Circulating progenitor cells from the bone marrow are also recruited to support new blood vessel formation. Appropriate synchronization of these cells is crucial for neovascularization and healing. However, population assays have been unsuccessful in definitively characterizing pericytes and circulating progenitor cells within the repairing wound.

In wound healing, active proliferation and reciprocal interactions of fibroblasts with other cell types in the wound environment, such as keratinocytes, endothelial cells, adipocytes, inflammatory cells, and resident stem cells, are important. Although reduced extracellular matrix deposition by fibroblasts can contribute to nonhealing wounds, excessive extracellular matrix deposition can lead to hypertrophic scarring and fibrosis [75]. Single-cell analyses have led to the identification of various fibroblast subpopulations with distinct functions after injury [7,76]. These technologies have identified unique subsets of fibroblasts that are responsible for the scar response.

Cellular Heterogeneity in Fibrosis

Tissue fibrosis is a common complication that underlies impaired tissue regeneration and tissue dysfunction in response to a variety of insults [6,75]. Fibrosis is a poorly understood process, but it is largely attributed to excessive extracellular matrix deposition by fibroblasts. However, fibroblasts are a heterogeneous population of cells [77]. To this end, single-cell technologies have been employed to interrogate fibroblast heterogeneity. It has been demonstrated that CD26+ fibroblasts constitute a distinct subpopulation of dermal fibroblasts, which is the primary cell type for excessive collagen deposition and scarring during wound healing associated with fibrosis [7]. Similarly, heterogeneity in fibroblasts mediating pathology such as pulmonary fibrosis and renal fibrosis have also been described in the literature [78,79]. Moreover, matrix stiffness cues from cross-linked collagen can induce other cells to turn into fibroblast-like cells, further contributing to fibroblast heterogeneity [80].

Macrophages are one of the cell types that deposit collagen in response to matrix stiffness. Thus, cellular heterogeneity in macrophage populations has formed the basis of many fibrosis studies. Traditionally, macrophages have been classified into proinflammatory M1 cells and antiinflammatory M2 cells [81,82]. Time-dependent shifts in relative proportions of M1/M2 macrophages underlie the reparative process as well as dysregulated excessive inflammation in the heart, kidney, and lungs. Comprehensive gene expression analysis of macrophages coupled with surface marker screening revealed that Ceacam1+/Msr1+/Ly6C−/F4/80−/Mac1+ cells, a distinct subpopulation of cells, is the chief contributor to bleomycin-induced fibrosis [83]. Similarly, subpopulations of macrophages that express CD34/CD68 have been found to be more prone to differentiate into myofibroblasts [84]. Single-cell transcriptional analysis has also been employed to study heterogeneity in fibrocytes, which are hematopoietic cells depositing collagen during tissue repair and fibrosis. This revealed the presence of a CD45+/CD11b+/F480+ macrophage subpopulation forming fibrocytes during wound healing [35]. Further research into tissue-specific cellular heterogeneity will help develop therapeutic strategies to control fibrosis. Enhanced understanding of cell heterogeneity in fibrosis could lead to strategies for cellular reprogramming, with implications in wound healing therapeutics, tissue engineering, and regenerative medicine [77].

Cellular Heterogeneity in Aging

Aging affects the regenerative capacity of most tissues. At the stem and progenitor cell level, these changes are attributed to both alterations of the intrinsic stem cell state and perturbations in the composition of stem cell subpopulations, which have been difficult to dissect in the past. Single-cell RNA seq has been used to differentiate between these cell-intrinsic and subpopulation differences in hematopoietic stem cells and progenitor cells. From a cell cycle perspective, these studies reveal a reduction in long-term HSCs (LT-HSCs) in the G1 phase with age. From a differentiation perspective, aged short-term-HSCs (ST-HSCs) resemble young LT-HSCs, which demonstrates that aged ST-HSCs fully self-renew and serve as the main source of hematopoietic maintenance in mice [85,86].

Single-cell qPCR has been used to evaluate the effects of aging on subcutaneous ASCs and influence their ability to support neovascularization in a wound healing setting. Although aging does not bring about changes in ASC

number, viability, or proliferative capacity, the single-cell study demonstrated that aging depletes a vasculogenic subpopulation of ASCs, leading to impairments in wound healing [63].

Single-cell RNA seq has been used to characterize adult NSC and progenitor cell populations, to determine heterogeneity within these cells. This resulted in the finding that NSCs can be clustered into early, mid-, and late-stage subpopulations along the stages of activation and differentiation. This study provides an integrative understanding of the NSC lineage with clinical implications in aging [87].

Tumor Cell Heterogeneity and Drug Resistance

Cells within tumors exhibit differential mutations and are derived from multiple lineages resulting in intratumor heterogeneity [52]. Tumor cell heterogeneity is a chief contributor to tumor invasion, metastasis, and resistance to drug therapy [52,88]. Two models have been proposed to drive this heterogeneity. The first, called the clonal evolution model, proposes that most neoplasms originate from a single cell, and the stepwise acquisition of mutations within this clone allows for the formation of more aggressive subclones, leading to tumor progression [89]. The second, theoretically opposing hypothesis, the cancer stem cell model, suggests that only a small subset of cells, called cancer stem cells, have tumorigenic potential, whereas their differentiated progeny have limited proliferation and tumorigenic potential. The elucidation of these two models had depended on xenograft limiting dilution assays and tumor markers from the literature [89]. Powerful single-cell technologies to discern this information had not yet been developed until now. These technologies might be able to suggest that the two tumor models are not mutually exclusive; cells within the tumor may display vast phenotypic plasticity and differentiated tumor cells undergoing dedifferentiation to acquire stemlike properties [90].

Single-cell RNA seq has been used to distinguish transcriptional diversity in genes regulating proliferation, immune response, and oncogenic signaling in cells isolated from human tumors [91]. In human glioblastomas, which is the most common and aggressive form of brain tumor, with an exceptionally low rate of survival, there has been a search for brain tumor−initiating cells. One study using single-cell qPCR identified a distinct DDR1+ subset from murine and human glioblastomas as the primary driver of aggressive tumorigenicity in vivo [34]. Although glioblastoma is a well-established example, there is limited information from other brain tumors. Single-cell RNA seq has been applied to study other brain tumors, such as from patients with oligodendroglioma, and has revealed stem/progenitor cell populations and unique differentiation programs within cells of these tumors [92].

In breast cancer, cancer stem cell subsets have been studied, with CD44high/CD24low cells representing a quiescent invasive mesenchymal state and ALDH+ cells representing a more proliferative epithelial state [93]. Advances in single-cell transcriptional profiling have taken these studies a step further and revealed novel targets such as calcium- and zinc-binding protein encoding gene (S100A9) to target breast cancer metastasis [94,95]. Similarly, single-cell sequencing of breast cancer cells has identified subpopulations that are resistant to chemotherapy. IGF1R+/KDM5A+/ITGA6+ breast cancer cells, for example, have been found to be resistant to drugs such as Paclitaxel [96]. In addition, in situ single-cell analysis suggested that chemotherapy before human epidermal growth factor receptor 2−targeted therapy can increase treatment resistance as a result of changes in intratumor diversity [97].

In a similar vein, single-cell RNA seq of lung adenocarcinoma cells revealed a unique subset of KRAS G12D+/high RS cells that are resistant to chemotherapy [98]. Single-cell analysis of human colon cancer samples identified cellular subsets with unique signatures such as $KRT20^{negative}$/$(CA1, MS4A12, CD177, SLC26A3)^{negative}$ that correlate with worse clinical prognosis [99]. Similar approaches have been used to identify POU5F1+ cells as a subpopulation of invasive cells in melanoma [100].

Therefore, new single-cell analysis techniques enable the identification of specific tumor cell subpopulations that are resistant to drugs, mediate tumor metastasis, and are responsible for tumor relapse. These novel targets can be used to develop targeted cell-specific treatments for cancer.

CONCLUSIONS

For a long time, our understanding of biology has been defined by measuring population averages of cellular behavior. However, in most cases, population averages do not result in accurate information, because cells within the same population exhibit heterogeneity. Rare but important cells such as stem and progenitor cells are almost unidentifiable in population-averaged studies. The evolution of single-cell technologies such as next-generation sequencing offers for the first time a comprehensive analysis of the entire genome and transcriptome of single cells,

and the ability to discover rare and previously unidentified cells. These technologies reveal changes within individual cells without previous bias from the literature. However, obtaining single-cell data is a challenging process in terms of time, resources, technical know-how, and the reliability of analyzed data. Moreover, a major concern that accompanies single-cell sequencing technologies is the presence of technical and biological noise that needs to be differentiated from biologic variation and heterogeneity. Whether these challenges can be overcome is doubtful. Thus, it is important to determine in what cases comprehensiveness of biological information is necessary. In clinical situations, particularly those in which patients must be diagnosed or in which therapeutic products must be used in patients, unreliability of data is unacceptable. In such situations, the use of accurate and precise technologies such as single-cell qPCR and FACS remains the norm. Platforms such as the Needlestack combine RNA seq, single-cell qPCR, and FACS to provide reliable single-cell readouts and validate the cellular subpopulations discovered. Development of such accurate and precise single technologies will aid in the fundamental understanding of human biology and guide therapeutic development.

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CHAPTER

7

Embryonic Stem Cells: Derivation, Properties, and Challenges

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INTRODUCTION

Embryonic stem cells (ESC) can be viewed as an immortal extension of short-lived pluripotent cells that exist in a preimplantation embryo. These pluripotent cells become all of the tissues of the body during embryo development, and cell lines created in vitro from these pluripotent cells retain important properties: self-renewal and the ability to differentiate into a variety of tissues of all three germ layers. An in vitro research model of these cells established in 1981 [1,2] immediately became indispensable for studying mechanisms of mammalian development, and when ESC were derived from human embryo in 1998 [3], regenerative medicine received a new and promising source of cells for tissue engineering.

Cells of any type intended for a therapeutic application have to be functional in vivo, nontumorigenic, and free of pathogens, and it is highly desirable to have a reliable long-lasting source of these cells. When such cells are isolated from donor tissues, their potential for expansion is limited, which restricts the use of this source. As a desirable off-the-shelf product, pluripotent cells seem to be an excellent source of differentiated derivatives: Their ability to self-renew allows for virtually limitless in vitro expansion, thus enabling large-scale manufacture, and they can be differentiated into a variety of derivatives that in turn can be purified and expanded.

DERIVATION OF EMBRYONIC STEM CELLS

Mouse Embryonic Stem Cells

In 1981, two independent research efforts resulted in the derivation of the first ESC lines from mouse embryos [1,2]. Both approaches used mitotically inactivated STO cells as feeders and based their assessment of cell morphology on the morphology of mouse embryonic carcinoma (EC) cells maintained in each laboratory. Evans and Kaufman considered critical factors for the success of derivation of pluripotent cell lines to be the window of embryonic development when pluripotent cells that would grow in culture existed in an embryo, the isolation of a sufficiently large number of such cells, and tissue culture conditions supportive of proliferation rather than differentiation of these cells. They first used an artificial delay in implantation induced by ovariectomy, which allowed late-stage embryos to remain free-floating in the uterus and to grow a large number of cells with no further development beyond primary ectoderm. After a 4- to 6-day delay, the blastocysts were recovered and cultured until egg cylinder-like structures formed that were isolated, trypsinized, and subcultured on mitotically inactivated STO cells, and selected for colonies resembling EC cells.

Martin based her work on the premise that teratocarcinoma stem cells are derived from pluripotent cells of the periimplantation embryo and produce pluripotency-supporting factors. She used conditioned medium from an established EC cell line as a source of such factors and plated the inner cell masses (ICMs) of mouse blastocysts isolated by immunosurgery [4] on the STO feeder layer in such conditioned medium. Resulting colonies with morphology resembling EC cells were selected and passaged until a high-density culture was established that no

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longer depended on conditioned medium, because ESC were probably making pluripotency-supporting factors themselves.

Pluripotent cell lines from both studies had a normal karyotype and differentiated into cells of three germ layers. These works laid the foundation for the huge field of study in cell and developmental biology on pluripotent stem cells and their differentiation.

Mouse ESC are typically derived from mouse blastocysts with optional immunosurgery. They can be cultured indefinitely on mitotically inactivated STO cells or mouse embryonic fibroblast feeder layers in the presence of leukemia inhibitory factor (LIF) and fetal bovine serum (FBS), they express several markers of pluripotency, and they have high alkaline phosphatase activity [5]. Common pluripotency markers are transcriptional factor Oct-3/4, originally reported in the ICM of an early blastocyst [6,7], as well as Nanog [8], Sox-2, Rex-1, Dnmt3b, Lin-28 [9], and cell surface antigen SSEA-1, which is expressed on the surface of blastomeres of the eight cell−stage embryo and ICM [9a]. ESC have become a common tool for generating transgene mouse models, because they easily aggregate with the ICM of a blastocyst when injected, or with blastomeres, making aggregation chimeras. ESC could also be generated from a single blastomere of a multicell-stage embryo [10] and appear to have the same properties including germ line transmission.

Human Embryonic Stem Cells

In 1998, in a groundbreaking report from the group led by James Thomson at the University of Wisconsin [3], the derivation of human ESC was announced. The team used an approach similar to that for deriving mouse ESC: An ICM of a blastocyst, isolated by immunosurgery, was plated onto mouse embryonic fibroblast feeder cells in medium supplemented with LIF, basic fibroblast growth factor (bFGF), and knockout serum replacement (KSR) (Life Technologies) in place of FBS. Several human ESC (hESC) lines were generated and soon became available for use by other researchers worldwide. hESC have the capability of differentiating into any and all cell types of the human body; they immediately became recognized as a promising source of many cell types sought after by regenerative medicine. However, this discovery also evoked great controversy and led to heated discussions in the media because some considered the destruction of a preimplantation human embryo to be the same as killing a human.

The use of federal funds for research involving hESC lines is allowed for National Institutes of Health−registered cell lines; there were 378 eligible hESC lines as of January, 2017 (https://grants.nih.gov/stem_cells/registry/current.htm). Other examples of stem cell registries are the University of Massachusetts International Stem Cell Registry http://www.umassmed.edu/iscr/index.aspx and the European Human Embryonic Stem Cell Registry http://www.hescreg.eu/.

ESC retain the fundamental property of ICM cells: the ability to give rise or develop into all tissues of the human body. The same markers of pluripotency are found in both ICM and hESC: transcription factors Oct-4, NANOG, and Rex-1; cell surface antigens SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81; and high endogenous alkaline phosphatase activity [11,12]. They retain high telomerase activity and can continue to self-renew indefinitely. Both in vitro and in vivo, when injected into immune-deficient mice, hESC form teratomas, tumors that contain derivatives of all three germ layers, with the most commonly seen ones being bone, cartilage, neural rosettes, and epithelium of the airways and gut.

SOURCES OF HUMAN EMBRYONIC STEM CELLS

Blastocyst

The first and most commonly used source of hESC is a blastocyst. Many groups noticed that fully expanded and spontaneously hatching blastocysts are not the best candidates for the derivation of ESC: their outgrowth is very prone to differentiation, probably because of some intrinsic commitments already made by the cells of the ICM. In addition, the growth of trophoblast cells can potentially overgrow ICM-originated cells. The latter can be avoided by performing immunosurgery [4], in which a zona pellucida−free blastocyst is incubated with antibodies that bind to the surface of trophectoderm, and then complement is added. This results in the lysis of trophoblast cells. The trophoblast-free ICM is plated for outgrowth. This procedure increases the efficiency of cell line derivation, but the success rate also depends on the quality and size of the ICM. Common impediments that may be encountered are spontaneous differentiation and apoptosis observed during the first week of ICM or blastocyst outgrowth. As mentioned earlier, Evans and Kaufman used delayed implantation of mouse embryos to achieve a larger ICM;

Stoikovic and coauthors used a similar approach. They cultured late-stage blastocysts in the presence of Buffalo rat liver (BRL) cell—conditioned medium through day 8, which allowed them to obtain larger ICM without its extensive further differentiation, and resulted in the successful derivation of hESC [13].

Morula

As an alternative to whole blastocyst, which represents a relatively late stage in preimplantation embryonic development when the specification of the cell fate starts, earlier-stage embryos (morulae) were successfully used [14] to create hESC lines with an efficiency comparable to that for ICM- or whole blastocyst—derived hESC lines. These hESC appeared to have the same properties as blastocyst-derived hESC, such as a pattern of expression of pluripotency markers, differentiation to all three germ layers, immortality in culture, and a normal karyotype.

Growth-Arrested Embryo

While many hESC cell lines have been created using leftover in vitro fertilization (IVF) embryos donated to research by couples undergoing infertility treatment, scientists kept working on alternative ways to make hESC without destroying the embryo. One of the first successfully executed approaches was the derivation of hESC lines from nonviable growth-arrested embryos [15,16]. This demonstrated that such embryos still have viable pluripotent cells that can be used to generate hESC lines.

Somatic Cell Nuclear Transfer

A highly publicized approach to hESC derivation is based on somatic cell nuclear transfer (SCNT). During a micromanipulation procedure, an unfertilized egg is enucleated and the nucleus of a donor cell is introduced into the egg via a micropipette. The egg with the donor nucleus then develops into a blastocyst that can be used to isolate ESC. Such ESC would have the same genotype as the donor of the nucleus and can be used to generate an autologous cell type for tissue repair. Several research groups [17,17a,18] succeeded in creating hESC by SCNT using fetal, neonatal, and adult fibroblasts as donor cells for somatic nuclei; the success rate was 25% [18a], which was comparable to that for hESC derivation from a naturally fertilized blastocyst. However, the efficiency of blastocyst formation and hESC derivation seemed to be different for eggs from different donors and can even correlate with the hormonal stimulation protocol [18]. In addition, it was discovered [18a] that a major SCNT reprogramming barrier was associated with the severe methylation of lysine 9 in histone H3 in a human somatic cell genome. The introduction of KDM4A, an H3K9me3 demethylase, during SCNT significantly improved the development and blastocyst formation of SCNT embryos, even when the authors deliberately used eggs from donors whose eggs had previously failed to produce SCNT blastocysts. Although the possibility of creating donor-matched hESC lines by SCNT remains attractive, it takes years for the operator to develop the skills required to perform this procedure with high precision and minimal disturbance to the egg or donor nucleus for successful outcome, so this approach to deriving hESC remains in the realm of only a few groups in the world.

Parthenogenesis

Another attractive possibility for generating pluripotent cells without destroying the embryos and overcome the problem of immune compatibility at the same time, is the generation of ESC and their derivatives from activated nonfertilized oocytes, or parthenotes [19], that would carry only maternal human leukocyte antigen (HLA) genes and thus allow to reduce the variability and number of lines required for immune match of the large number of patients. Due to genetic imprinting and the deficiencies of maternal and paternal haploid gene sets, their combined action is required for normal development. Parthenote mammalian embryos which do not have paternal genes are unable to develop to term; however, pluripotent cells produced from such parthenote human embryos seem to have phenotypes, behavior, and differentiation potential similar to those of hESC from blastocysts [20—23]. From an ethical viewpoint, parthenote hESC may be less controversial because no life is destroyed [23a]. A lot of progress has been made in generating human parthenote ESC and their derivatives [24—29], but data on the behavior of such derivatives in vivo are still limited. Several studies showed that a large percentage of parthenogenetic blastomeres was affected by an excessive number of centrioles, a high aneuploidy rate [30], and genetic and epigenetic

instability [31]. More studies demonstrating the safety and efficacy of parthenote hESC derivatives are needed before this attractive source of HLA-matched cells for regenerative medicine can be fully used.

Single Blastomere

To address the ethical controversy of hESC derivation from blastocysts, our group developed an approach based on single blastomere biopsy, a procedure commonly employed in preimplantation genetic diagnostics (PGD) in the course of IVF [32–34]. In this procedure, a hole is made in the zona pellucida of a morula stage embryo using acid Tyrode solution or with the help of a laser, and a single blastomere is extracted. The embryo continues to develop while the blastomere undergoes PGD tests, and a few days later, blastocysts that were "cleared" can be implanted. This procedure has been deemed safe and has resulted in hundreds of healthy babies being born. Using blastomere biopsy, we established several hESC lines from single blastomeres, whereas the parent embryos were allowed to develop to the blastocyst stage and then were cryopreserved [35]. Single blastomeres were first cocultured with the biopsied embryos and then were plated onto feeder cells in microdrops. Outgrowing colonies were treated in the same way as ICM outgrowths: with careful mechanical passaging followed by enzymatic dissociation when an appropriate number of colonies could be achieved. Established hESC lines had properties similar to ICM-derived hESC: they stained positively for Oct-4, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase; they had a normal karyotype and differentiated into derivatives of all three germ layers both in teratoma assays in nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice and in vitro. The success rates for hESC derivation from blastocysts and blastomeres can be similar, around 25%–30%. This technique was subsequently used by several other groups to derive hESC lines with high efficiency and/or without destroying the embryo under different conditions including human feeder cells, feeder-free, and even from growth-arrested embryos [36–41]. Single blastomere–derived hESC showed a transcriptional profile similar to "conventional" hESC [38], and several differentiated derivatives of single blastomere–hESC appeared functional in vitro and in vivo [42,43,43a]. By avoiding the destruction of human embryos, this approach has offered a way to address ethical concerns regarding hESC derivation and provided a way to overcome a major impediment in developing hESC-based therapies.

HUMAN EMBRYONIC STEM CELL MAINTENANCE

Similar to early mammalian development, when multiple cell–cell and cell–matrix interaction produce a variety of both inductive and permissive differentiation signals, ESC culture reproduces such signals with a certain degree of approximation: hESC readily differentiate into all three lineages, but the efficiency of their commitment to each lineage often depends on specific culture conditions, because this artificial system does not provide the same finely tuned orchestration as that which happens during in vivo pattern formation. The strong inclination of hESC to differentiate in culture makes it challenging to maintain hESC in a self-renewing state and is associated with the limitations of a two-dimensional cell culture environment and media components used as substitutes for the short-lived microenvironment of a preimplantation embryo. Meticulous attention to several key factors is required for the successful maintenance of hESC in a pluripotent state for multiple passages:

- Microenvironment: defined combination of media and matrix, with or without feeder cells
- Maintenance: frequent passaging using gentle and efficient cell–matrix disruption
- Morphology observation performed daily to assess the culture and avoid subculturing cells with signs of differentiation

Microenvironment

hESC are usually cultured on either a monolayer of mitotically inactivated feeder cells or a defined extracellular matrix (for example, Matrigel, vitronectin, laminin-521) in defined media that allow them to be propagated in culture virtually indefinitely, unlike cells of the ICM, which exist only transiently. Like the ICM of a developing blastocyst, which is programmed to differentiate once the implantation starts, pluripotent ESC in culture readily differentiate when the microenvironment changes. Even the most carefully selected and tested combinations of media and matrix will not allow hESC to keep growing in the same dish and remain pluripotent indefinitely, because when colonies

reach a certain size and become overcrowded, they form a second layer of cells, and then the cells in the colony begin to lose pluripotent marker expression and differentiate. Timely passaging prevents this loss of pluripotency because it releases single cells and small cell clumps, so any matrix–cell interactions that were formed during the first days after previous passage and begin to send differentiation signals to hESC are disrupted, and the cells can continue to self-renew.

The first hESC lines were derived and cultured under conditions similar to what is used for derivation and maintenance of mouse ESC: mitotically inactivated mouse embryonic fibroblasts (MEF) plated on gelatin, in a medium based on KSR, a proprietary serum substitute formulation. This medium was supplemented with LIF and bFGF [3,11,33,34]. Variations of this system include adding Plasmanate [33] or using a 1:1 mix of Knockout- Dulbecco's Modified Eagle Medium and F12 medium. However, it was reported that human hESC do not have an active signal transducer and activator of transcription 3 (STAT3) pathway and thus are LIF-independent [44,45], and many researchers stopped using it in the culture medium. Indeed, it appeared that using bFGF alone is sufficient to support the pluripotency of hESC over multiple passages. Other studies have shown the importance of LIF in maintaining hESC in what is called a naive state (discussed subsequently), so it is probably too early to make a conclusion about the need of this factor to maintain pluripotency.

Maintenance

It is commonly observed that cultures of hESC contain differentiating cells that, when there are only few of them, usually do not interfere with the successful maintenance of hESC in a pluripotent state or with their differentiation toward a desired derivative. However, if the colonies are allowed to overgrow, soon afterward they become multilayered or begin to touch each other, and spontaneous differentiation usually follows within hours. Although it is possible to rescue even extensively differentiated cultures (for instance, by carefully selecting undifferentiated colony pieces, or "mechanical picking," it is more practical to prevent the loss of pluripotency by timely passaging based on observing the colony morphology and confluency and by using high-quality reagents and good cell handling practices.

For an hESC subculture, there is a wide variety of commercially available dissociating agents. In the past, collagenase used by Thomson and colleagues [3] for hESC derivation and passaging was an enzyme of choice for many laboratories. It allowed the cells to be passaged as clumps rather than as single cells, but it required meticulous attention to colony morphology because when colonies of larger size are harvested, they are more prone to spontaneous differentiation. Trypsin is another popular enzyme, but because of its rapid action, it demands careful techniques during cell harvest to avoid cell damage and death. Mechanical colony dispersion and hand picking allows the selection of colonies of "proper" morphology with minimal stress to the cells and is the most commonly used method to derive new lines (when the outgrowth is small and the removal of differentiating cells is needed to prevent further differentiation of the remaining pluripotent cells). However, this method is operator-biased and demands sufficient experience to avoid selecting for aneuploid cells that may have a growth advantage and thus are the first to form good-sized and "good-looking" colonies. On the other hand, a skilled operator may be able to rescue an aneuploid culture by carefully selecting and dispersing colonies of the right morphology. Other available dissociation methods that allow hESC colonies to gently break into small cell clumps are Accutase, TrypLE (proprietary mix of enzymes, Life Technologies), and "Dissociation Buffer" (containing chelating agents, Life Technologies).

hESC can maintain normal karyotype over multiple passages, but they are prone to aneuploidy. There are not enough data to identify reliably which factors cause aneuploidy, although a study showed an association of aneuploidy with high-density culture [46]. The authors demonstrated that 33% of hESC became genetically abnormal after only 5 days of high-density culture. It seemed that lactic acid and acidification of the medium were the main reason for such abnormalities, but interestingly, laminin 521 used as matrix counteracted this effect. Frequent change of medium also prevented such aberrations. Some of most commonly seen chromosome abnormalities, such as trisomy in chromosome 12 or 17 [47], are known to result in hESC survival or growth advantage, so even an initial nonclonal aberration can quickly become prevalent. Other common abnormalities in hESC karyotype are seen with chromosomes 1, 14, and 20 [48]. Dissociating agents can contribute to the quick spread of abnormal cells throughout the population [48a]. This tendency of hESC to undergo clonal aneuploidy reinforces the importance of making frequent karyotyping a part of the routine maintenance of hESC. G-banding with the examination of a minimum of 20 cells complemented by fluorescence in situ hybridization (FISH) with probes for chromosomes 1, 4, 12, 17, and 20 can be considered appropriate karyotyping methods.

When choosing the most suitable combination of matrix, media, and passaging methods, it is important to remember that in addition to supporting pluripotency, a stable karyotype and reproducibility, the culture system needs to ensure the acceptable efficiency of the differentiation of hESC into the desired derivative under specific protocols. For instance, the formation of embryoid bodies (EB) (plating hESC into low-attachment cell culture plates, where they can aggregate and form cell clumps, differentiating into three germ layers) is frequently used to simulate early differentiation events in mammalian development. However, when hESC are dissociated into single cells, the formation of EB may be impeded by low cell survival, and the yields of EBs and differentiated cells can be much lower [48b].

Morphology

The morphology of individual hESC and of colonies was mentioned earlier with regard to the maintenance routine and the choice of culture conditions. hESC have been known to form colonies with sharp "shiny" borders when they are cultured on feeder cells, and cells in such colonies are small with a high ratio of nuclei to cytoplasts and visible nucleoli. When cultured feeder-free, hESC look larger and more spread-out [55]. The borders of the colonies may become sharp after only several days in culture, when the cells become almost "overgrown." Under these conditions, hESC inside the colonies look relatively large and flat for the first few days and may even resemble early stages of differentiation, which can be confusing to an inexperienced eye; however, after several days, the morphology of such colonies becomes more similar to typical hESC as cells become "packed," so they become smaller in diameter. However, larger and flatter cells can also indicate the beginning of differentiation, and daily follow-up of the same cell culture dish is important to better understand the nuances of cell morphology, to tell apart which deviations from the "ideal" morphology are associated with cell adaptation after passaging and which are early signs of differentiation.

Evolution of Human Embryonic Stem Cell Derivation and Culture Methods

There are several commercially available media with a proprietary blend of growth factors and nutrients that support the maintenance of hESC growth and pluripotency. Some examples are NutriStem (Biological Industries, Israel), TeSR media (Stem Cell Technologies), and E8 (Thermo Fisher Scientific). These media usually come with a detailed protocol prompting the researcher to use a certain matrix−media combination. Commonly used extracellular matrices are Matrigel, an extracellular matrix produced by Engelbreth-Holm-Swarm mouse sarcoma cells (BD Biosciences), laminin 521 or 511 (BioLamina Sweden), CELLstart, a human placenta-derived extracellular matrix (Life Technologies), and vitronectin. If a special matrix−media combination is desired, the conditions have to be carefully tested: hESC need to be subcultured for several passages under new conditions before it is known whether a certain combination works for the support of growth and pluripotency and ensures karyotype stability. MEF as feeder cells have been a reference standard for ESC culture for years. Since the beginning of hESC research, there has been great interest in using their derivatives clinically, but coculture of live human cells with live animal cells results in a xenogeneic product and requires more extensive testing for animal viruses. Thus is has been highly desirable to be able to derive and propagate hESC without feeder cells of animal origin or even make them feeder-free. Although xenogeneic cell products are allowed for transplantation in human patients, there are more stringent regulations. For instance, the patient's blood samples needs to be archived for a prolonged time, which adds to the costs and logistics of clinical applications. The first reports on using feeder cells of human origin demonstrated the feasibility of using cells from different donors and/or different tissues, although some cell types seemed to be more effective in supporting hESC derivation and growth [39,49−53].

Another step in the transition from a xenogeneic to a nonxenogeneic cell product was the feeder-free culture system [54], in which the MEF-conditioned cell culture medium allowed hESC to propagate on Matrigel or laminin and retained their pluripotency and normal karyotype. However, this work was done using cell lines established with live mouse feeder cells, so these feeder-free cultured hESC still classified as a xenogeneic product. We attempted to derive new cell lines from blastocysts that were completely feeder-free. We postulated that an important factor in establishing the outgrowth of pluripotent cells was interaction with an extracellular matrix that was produced and properly organized by feeder cells. MEF were cultured for several days to allow for this matrix to be produced and organized, and then sodium deoxycholate was used to lyse the MEF whereas the preassembled extracellular matrix was left intact. The blastocysts or ICMs were plated onto this matrix in KSR-based derivation medium supplemented with LIF and bFGF, and the outgrowing hESC colonies were dispersed using mechanical passaging or

trypsin–EDTA. hESC cell lines that showed normal karyotype, maintained pluripotency marker expression over multiple passages and differentiated into derivatives of all three germ layers in vivo and in vitro was established as a result of this work [55]. Although derivation of hESC is more challenging than propagation, more feeder-free hESC lines were established using novel defined media such as NutriStem [40].

NAIVE EMBRYONIC STEM CELLS

hESC and mouse ESC have many similarities, yet they are different. Mouse ESC have been shown to exist in two states: "naive" and "primed" [56–59]. Not only do naive pluripotent cells express markers of pluripotency such as Oct4, NANOG, Sox2, both X-chromosomes are active. Primed ESC coming from the epiblast (EpiSC) retain the expression of these markers, but only one X chromosome is active [60]. hESC resemble EpiSC rather than naive: the colonies they form are flat, not dome-like as is typical for naive cells, they are polarized [60a]; their metabolism is mostly glycolic whereas primed cells rely on oxidative phosphorylation [61,62]; only one X-chromosome is active; and they are sensitive to single-cell dissociation [57,63]. Naive cells survive single-cell dissociation much better, which allows for greater capacity for expansion (which could be highly desirable for large-scale manufacturing), their doubling time is much shorter, and they have been shown to differentiate more efficiently both in vivo and in vitro [64,65], a highly sought-after property when differentiation is aimed at making derivatives for regenerative medicine.

Mouse ESC are usually derived and exist in a naive state, and it takes special effort and culture conditions to isolate EpiSC from an epiblast. On the other hand, most hESC lines were derived and propagated in a primed state, until naive, or "ground state" pluripotent hESC were derived [66,67]. It has been shown that naive hESC can be derived de novo, or existing hESC lines can be converted into the naive state. In both cases, signaling pathways need to be activated or inhibited using a cocktail of bioactive substances and small molecules. Some examples are MEK inhibitor PD0325901, GSK inhibitor CHIR99021, STAT3 inhibitor NSC74859, ROCK inhibitor Y27632, LIF, FGF2, and TGFβ1. Interestingly, all such cocktails include LIF, which was once considered to be essential for hESC maintenance in the pluripotent state but then was abandoned after it was shown that hESC do not depend on it for pluripotency. It remains unclear whether the transition from naive to primed hESC happens during the first days, if not hours, of derivation from a naive ICM or whether such primed cells exist in the embryo. RNA sequencing analysis of single cells of human blastocysts showed that at least three types of cells can be identified, and that during derivation of hESC there are changes in gene expression as the cells adapt to culture conditions [68].

HUMAN EMBRYONIC STEM CELL DIFFERENTIATION AND MANUFACTURING FOR CLINICAL APPLICATION

Although hESC provide exciting research opportunities for studies related to the mechanisms of development, their biggest promise is in their differentiation potential. There are unmet medical needs for a variety of cell types, including but not limited to pancreatic β cells, hepatocytes, dopamine neurons, oligodendrocytes, retinal cells, cardiomyocytes, vascular cells, cartilage, and bone: derivatives of all three germ layers. Some of these cell types are frequently seen in hESC cultures left to differentiate spontaneously or in EB, and some require specialized derivation protocols for efficient yields. For instance, the neural path seems to be a default choice of ESC in the absence of other differentiating cues [69], and in spontaneously differentiating hESC cultures [69–71] the presence of various neural lineage cells is common. Perhaps this natural tendency of hESC to form derivatives of neural lineage endorsed derivation and studies of such derivatives, and oligodendrocyte progenitors were the first hESC-derived cells to enter clinical trials for spinal cord injury. More clinical trials followed using another hESC-derived progeny of the neural lineage: retinal pigment epithelium (RPE) cells to treat macular degeneration and Stargardt's disease in the United States and European Union [72,73].

RPE has a unique function: it provides support for the photoreceptor by delivering nutrients and removing shed outer segments. Its morphology is also unique which allow to easily detect these cells as pigmented cobblestone "islets" among various types of differentiating hESC. Unlike many terminally differentiated cell types, RPE retains its proliferation potential, so these pigmented epithelial cells can be relatively easily isolated with high purity and also be efficiently scaled up (Klimanskaya et al., 2004). Such hESC-derived RPE cells can fully differentiate and mature

after transplantation in animal models, fully integrate into the host's RPE layer, retain RPE morphology and molecular markers, and provide photoreceptor rescue [43a,73,74].

For this unique type of cells, a relatively small scale hESC culture can result in efficient production of the final product, and considering the small size of the macula and low cell numbers required for injection (in the range of several hundred thousand cells) [72,73], a relatively small-scale cell culture is needed to produce therapeutic doses. However, for other cell types considered for therapeutic applications that cannot undergo multiple population doublings while maintaining potency, or when large numbers of cells are required, various challenges can arise, such as using alternative cell culture systems including suspension culture, microcarrier- or microfluidics-based bioreactors at the hESC stage of the process, or isolation of progenitor type cells that can be expanded further.

Whereas there may be a variety of approaches to manufacturing different hESC-originated cell types, the same principles can be applied to producing all hESC derivatives to ensure their safety and efficacy. The US Food and Drug Administration issued a document on tissue donor regulations (*Guidance for Industry: Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)*, https://www.fda.gov/downloads/biologicsbloodvaccines/guidancecomplianceregulatoryinformation/guidances/tissue/ucm091345.pdf), and these regulations apply to embryo donors. All raw materials used in manufacturing need to meet the safety criteria and be fully characterized. Standard tests performed on manufactured cells include sterility, mycoplasma, common and latent viruses, and endotoxins. Of utmost importance is the safety of the cell product. First, there should be solid proof that the manufacturing process does not result in the presence of residual pluripotent cells in the final product, which can lead to the formation of teratoma, and the assays used to detect such pluripotent cells have to have a high level of sensitivity. Animal studies may be required to confirm that transplantation of the hESC derivative does not lead to tumor formation in immune-suppressed animals. Cell growth in soft agar can be used as an additional in vitro tumorigenicity test, and an assay should be in place to confirm the desirable purity of the derivative. Cells intended for transplantation have to have a normal karyotype confirmed by a rigorous test. The potency of the final product has to be confirmed using physiologically relevant assays: For instance, it could be phagocytosis for RPE, glucose-responsive insulin production for pancreatic β-cells, and bone and cartilage formation for mesenchymal stem cells. For each cell type, the most relevant and technically feasible in vitro assay should be chosen.

CONCLUSIONS

ESC help to recapitulate early events in mammalian development and are an excellent system to study self-renewal and differentiation. Since hESC were derived, numerous studies have demonstrated that they could be a promising source of various cell types for regenerative medicine. There are several alternative sources of pluripotent stem cells: the ICM of a blastocyst, morula, single blastomere, parthenote embryos, embryos generated via SCNT, growth-arrested embryos, and induced pluripotent cells. The same principles established through many years of research using ESC can be applied to the culture and differentiation of all pluripotent cell lines of different origins and sources to ensure their robust propagation in self-renewal state and efficient differentiation. Since the first derivation of hESC the culture methods have been become increasingly more robust, and there is now a variety of approaches to propagating pluripotent stem cells including hESC. Human feeder cells or feeder-free conditions can be used along with several defined media−extracellular matrix combinations and a variety of dissociating agents, including xenogeneic-free reagents.

At the same time, many challenges are associated with our incomplete knowledge of the fine mechanisms of self-renewal and differentiation. Further studies should help us to better understand which components of the cell culture system and steps in the maintenance routine are most critical in supporting hESC self-renewal, a stable karyotype, and differentiation efficiency to advance the use of hESC derivatives in regenerative medicine.

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CHAPTER

8

Alternative Sources of Human Embryonic Stem Cells

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INTRODUCTION

Human embryonic stem cells (hESC) are conventionally derived from viable preimplantation embryos produced by embryonic stem (IVF) [1]. The derivation of hESC is considered ethically controversial because of the typical destruction of the embryo during this process [2–5]. A human embryo constitutes an object of moral concern [6] owing to its identity as a human at the embryonic stage of development. In biological terms, a human embryo has a distinct, unique, and unambiguous status as a result of this identity. However, the political and moral status of human embryos are in a state of flux. Whereas there is universal opposition to reproductive cloning of humans by any method, there is diversity in opinion regarding the use of human embryos to derive hESC and, subsequently, potential therapies derived from them [7,8]. Ethical and cultural imperatives to respect human dignity from the moment of fertilization conflict with a utilitarian desire to relieve human suffering, even when this comes at the expense of embryonic human life. These conflicting perspectives have fueled an intense debate and have influenced legislative regulation of stem cell research in the United States and internationally [2–5,9,10]. US stem cell research policy was regulated on the federal level by the Dickey Amendment and President Obama's Executive Order 13,505 and by individual state laws (Box 8.1) [10]. The use of federal funding to derive new hESC that would entail destroying human embryos is forbidden. Also, in many European countries (Austria, Germany, Ireland, Italy, Lithuania, Norway, Poland, and Slovakia), the derivation of hESC from surplus embryos is prohibited [9]. Because stem cell biology is at the forefront of research, legislative acts change rapidly. (For up-to-date legislative regulation of human embryonic stem (ES) cell research, refer to links provided in Box 8.2) [9,10].

Another consideration is the constant demand to derive new human embryonic stem (ES) lines for both basic and clinical applications owing to the loss of genetic and epigenetic stability arising during hESC culture and manipulation [11–14]. Many available hESC lines had been exposed to animal material during derivation or culture [2,15]. It is acceptable to expose hESC lines to products of human origin, but it remains the ultimate goal to pursue hESC derivation under stringent xenogeneic-free conditions for eventual clinical use [2,15].

The debate on embryo-destructive derivation of ES cells often focuses on the moral sensibilities of investigators and their desires for research unfettered by ethical considerations. However, the goal of hESC research is to find therapies that would ease human pain or debilitation caused by illness or injury [2,16,17]. In the latter context, the sensibilities of many millions of the populace (the intended beneficiaries of this work) should be instructive. As a result, a variety of different derivation strategies have been proposed (Fig. 8.1) to avoid using an embryo as a source of human stem cells (detailed information can be found in appropriate chapters of this book or elsewhere) [2,3]. In this chapter, we will discuss two alternative approaches to yielding genetically unmodified hESC that do not interfere with the developmental potential of human embryos: single blastomere biopsy (SBB) and organismically dead embryos (Fig. 8.1) [2].

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BOX 8.1

BRIEF OVERVIEW OF US FEDERAL STEM CELL POLICY

The US policy on stem cell research is shaped by the following legislative act and executive order:

- The "Dickey amendment," a rider issued in 1996 that framed all subsequent political discussions regarding human embryonic stem cells (hESC) research. The amendment stated that no federal funding may be employed for (1) the creation of a human embryo or embryos for research purposes or (2) research in which a human embryo or embryos are destroyed, discarded, or knowingly subjected to risk of injury or death (beyond that permitted for fetuses in utero under the Public Health Service Act).
- Executive Order (EO) 13,505, which removed barriers to responsible scientific research involving human stem cells. This EO was issued by President Obama on March 9, 2009 and stated that the Secretary of Health and Human Services, through the director of the National Institutes of Health, may support and conduct responsible, scientifically worthy human stem cell research, including human stem cell research, to the extent permitted by law. In addition, this EO revoked two items issued by President George W. Bush: (1) a presidential statement that permitted work only on hESC lines generated before August 9, 2001, and (2) EO 13,435, which favored all research on stem cells without harming a human embryo.

BOX 8.2

USEFUL LINKS AND RESOURCES FOR INFORMATION ON CURRENT LEGISLATION IN THE UNITED STATES AND INTERNATIONALLY

National Institutes of Health (NIH) Stem Cell Information webpage: contains relevant information on current US stem cell policy; NIH Stem Cell Registry with a list of eligible lines for NIH funding. The page also contains public comments on draft NIH human stem cell guidelines that supplement Executive Order 13,505. http://stemcells.nih.gov/index.asp.

International Society for Stem Cell Research webpage: contains comprehensive information on international legislation on human embryonic stem cell research; periodically updated. http://www.isscr.org/.

Single Blastomere Biopsy

SBB for the purpose of deriving ES cells was developed by Lanza and colleagues [18–21]. HESC are created from a single blastomere that is removed from the embryo [20,21] by employing a technique that was originally developed for preimplantation genetic diagnosis (PGD) [2,22–24]. This procedure bypasses the ethical issue of embryo destruction, because biopsied embryos continue to develop and reach the blastocyst stage and beyond, as demonstrated by more than a decade of experience with PGD [2,24]. SBB of both murine and human eight-cell stage embryos has been used successfully as a source of material to derive ES cell lines (Fig. 8.1) [2,18–21]. The risk associated with embryo biopsy [25] is accepted by patients as part of the PGD procedure, but it would be considered unjustified in a research setting in the absence of a clinical indication [2]. In addition, US regulations forbid research on an embryo that imposes greater than minimal risk, unless the research is for the direct benefit of the fetus (Box 8.1) [26]. To date, none of the hESC lines derived by SBB have been approved for National Institutes of Health (NIH) funding [10].

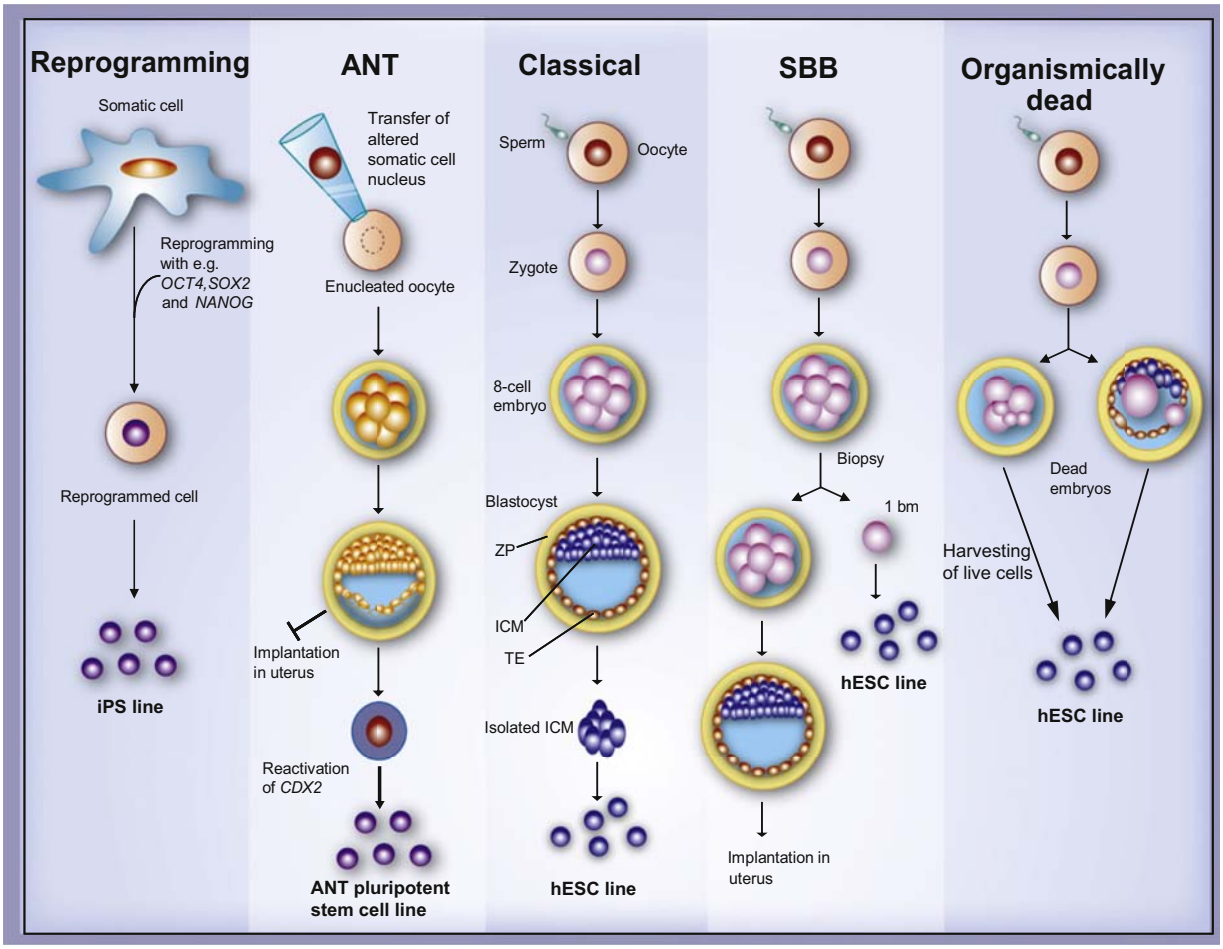

FIGURE 8.1 Classical and alternative strategies for the generation of human stem cells by reprogramming with exogenous genes (iPS), transfer of a genetically altered somatic cell nucleus into an oocyte (ANT), the classical derivation of human embryonic stem cells (hESCs) from blastocyst culture, the derivation of hESCs from a biopsied single blastomere (SBB), and the derivation from organismically dead embryos. *bm*, blastomere; *ICM*, inner cell mass; *iPS*, induced pluripotent stem cells; *TE*, trophectoderm; *ZP*, zona pellucida. *Reproduced with permission from Gavrilov S, Papaioannou VE, Landry DW. Alternative strategies for the derivation of human embryonic stem cell lines and the role of dead embryos. Curr Stem Cell Res Ther 2009a;4:81–6.*

ORGANISMICALLY DEAD EMBRYOS

Our group proposed the derivation of hESC from irreversibly arrested, nonviable human embryos that died, despite best efforts, during the course of IVF for reproductive purposes [2]. This proposal to harvest live cells from dead embryos is analogous to the harvesting of essential organs from deceased donors. We suggested that the established ethical guidelines for essential organ donation could be employed for the clinical application of this paradigm to generate new hESC lines [2,4,27,28].

Irreversibility as a Criterion for Diagnosing Embryonic Death

The modern concept of death is based on an irreversible loss of integrated organismic function [28,29]. Brain death is used as a reliable marker for irreversible loss of integrated function. Diagnosing the death of a patient before the

death of that patient's tissues is important for the appropriate application of medical resources and for the possibility of organ donation.

To apply this concept to a stage of development that precedes the development of the nervous system, we proposed that an irreversible arrest of cell division would mark an irreversible loss of integrated function. Thus, it was necessary to find criteria that would establish irreversible cessation of normal embryonic development before every cell of the embryo has died. Through retrospective analysis of early-stage embryos that had been generated for reproductive purpose but were rejected owing to poor quality and/or developmental arrest, we showed that many of these embryos were in fact organismically dead [28]. Our data showed that the failure of normal cell division for 48 h was irreversible, and despite the possible presence of individual living cells, they indicated an irreversible loss of integrated organismic function: the conceptual definition of death [2,28].

Furthermore, we conducted a prospective study to characterize embryonic death [3,30], in which the progression of arrested embryos, including abnormal blastocysts, was examined in extended culture [27]. Our data demonstrated that developmental arrest observed in some human embryos by embryonic day 6 (ED6) after IVF cannot be reversed by extended culture in conditions suitable for preimplantation embryos, because we saw no morphological changes indicative of developmental progression in most embryos and observed no unequivocal instances of further cell divisions [27]. Moreover, these observations are in line with standard IVF practice, which dictates that such embryos should not be transferred or cryopreserved because they are known not to produce live offspring [27,31–38]. In an attempt to correlate morphology with cell number, we categorized the embryos at ED6 on the basis of gross morphology (Fig. 8.2) [27]. We showed that morphological categorization was of limited value in predicting cell number. Nevertheless, the higher cell number associated with cavitation might predict greater potential for the success of hESC derivation [27]. In addition, we determined the proportion of living and nonliving cells in nonviable ED6 human embryos (Fig. 8.2) and showed that most irreversibly arrested embryos contain a high proportion of vital cells regardless of the stage of arrest, which indicates that harvesting cells and deriving hESC from such nonviable embryos should be feasible [27].

Human Embryonic Stem Cell Lines Derived From Irreversibly Arrested, Nonviable Embryos

In fact, the proof of principle for this alternative method has been obtained, because 14 hESC lines were successfully derived from nonviable embryos that were irreversibly arrested by our criteria (Table 8.1) [39,40,41]. The first cell line (hES-NCL9) was derived by Stojkovic and colleagues from 132 arrested embryos [40]. Subsequently, Daley and colleagues derived 11 lines from 413 poor-quality embryos rejected for clinical use [39]. In addition, our group derived two human ES lines: CU1 and CU2 from 159 ED6 irreversibly arrested, nonviable human embryos [41]. Although many arrested embryos might be expected to be aneuploid [42–46], all 14 hESC lines were karyotypically normal; moreover, pluripotency and differentiation potential were demonstrated in vitro and/or in vivo [39,40]; [41].

Morphological Criteria for Predicting the Capacity of Irreversibly Arrested, Nonviable Human Embryos to Develop Into a Human Embryonic Stem Cell Line

To define morphological criteria that could be used to predict the capacity of discarded, irreversibly arrested, nonviable embryos to develop into an hESC line, we carried out a retrospective analysis of the morphological progression from ED5 to ED6 in 2480 embryos that were rejected for clinical use [41]. Embryos were given a morphological category commonly used for clinical grading as per standard IVF practice (e.g., single-celled embryo, multicell, morula, blastocyst). If an embryo had reached the blastocyst stage (i.e., showing advanced cavitation), it was given an overall grade of good, fair, or poor, and was also scored for inner cell mass and trophectoderm quality. Our analysis showed that nonviable embryos defined as poor did not improve with extended in vitro culture but retained the capacity to yield hESC lines despite arrested development [41]. We postulated that if derivation efforts were targeted on this subgroup, the derivation success rate could be increased and the production of new hESC lines could be brought closer to clinical application [41].

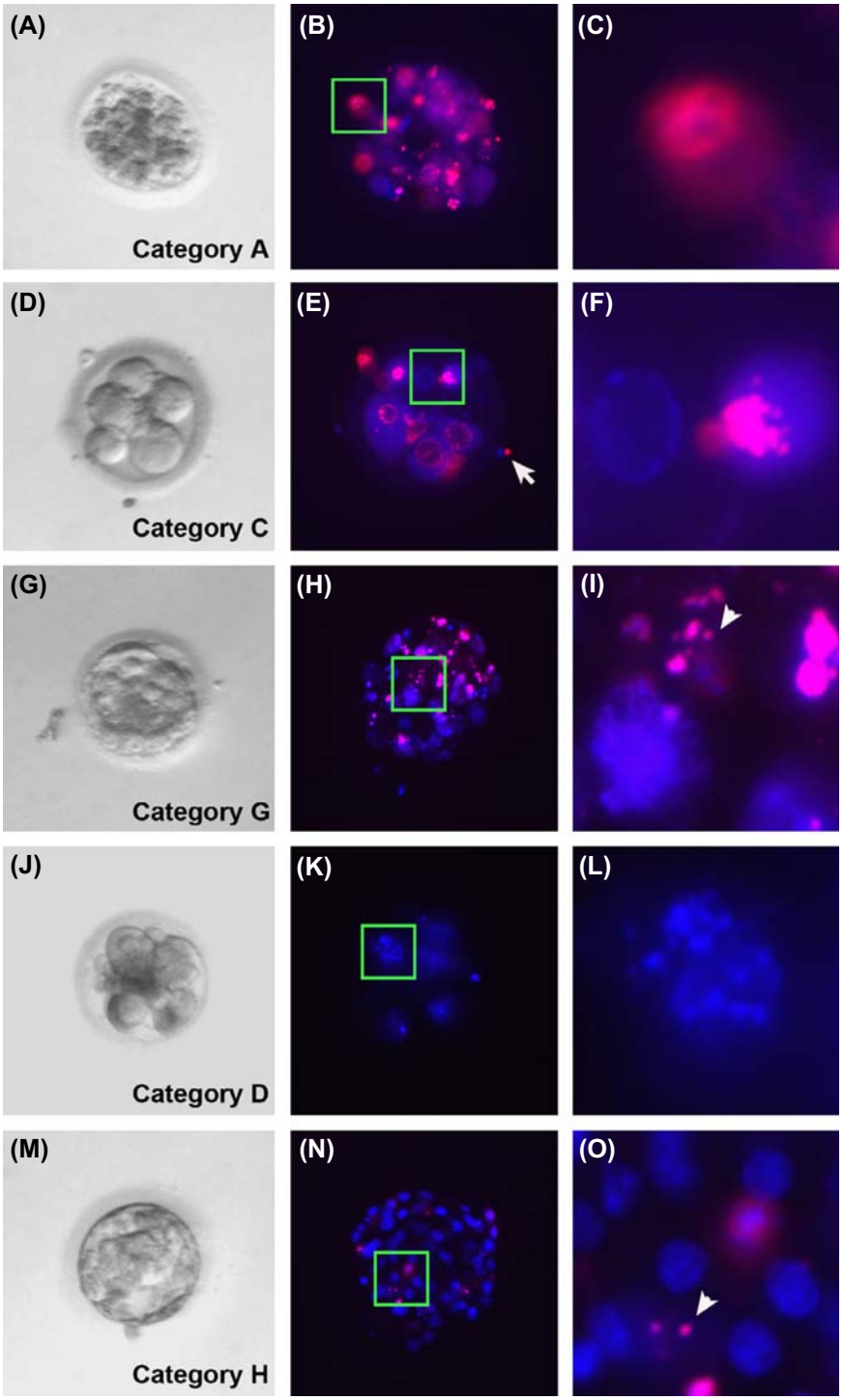

FIGURE 8.2 **Morphology and differential propidium iodide/Hoechst fluorescent nuclear staining of nonviable embryos at ED6.** Brightfield images (A, D, G, J, and M) with corresponding fluorescence images (B, E, H, K, and N), and enlarged details (C, F, I, L, and O) as indicated by the green squares (A–C). Category A embryo showing degeneration at embryonic day 6. All nuclei, including nuclear fragments, are pink, indicating that there are no living cells in the embryo. Detail shows pink nucleus from a dead cell (D–F). Category C embryo with living and dead cells is indicated by the blue and pink nuclei, respectively. Detail shows nuclei from one living and one dead cell. Arrow in E indicates a sperm nucleus outside the zona pellucida. (G–I) Category G embryo with living and dead cells as well as fragmented nuclei. Detail shows intact and fragmented nuclei (J–L). Category D embryo with all live cells. Detail shows blue fragmented nucleus (M–O). Category H embryo with many living and a few dead cells. Arrowheads in I and O indicate nuclear fragments. *Reproduced with permission from Gavrilov S, Prosser RW, Khalid I, MacDonald J, Sauer MV, Landry DW et al. Non-viable human embryos as a source of viable cells for embryonic stem cell derivation. Reprod Biomed Online 2009b;18:301–8.*

TABLE 8.1 List of Human Embryonic Stem Cell Lines Derived From Nonviable Organismically Dead Embryos

Cell Line Name	Type of Embryo	Karyotype	Stem Cell Markers	Embryoid Body Assay	Teratoma	Eligible for National Institutes of Health Funding?	References
hES-NCL9	Day 6–7 late arrested embryo (16–24 cells)	46,XX	Yes	Yes	Yes	ND	[40]
CHB-1	Day 3 PQE	46,XY	Yes	NR	Yes	Yes	[39]
CHB-2	Day 5 PQE	46,XX	Yes	NR	Yes	Yes	[39]
CHB-3	Day 5 PQE	46,XX	Yes	NR	Yes	Yes	[39]
CHB-4	Day 5 PQE	46,XY	Yes	NR	Yes	Yes	[39]
CHB-5	Day 5 PQE	46,XX	Yes	NR	Yes	Yes	[39]
CHB-6	Day 5 PQE	46,XX	Yes	NR	Yes	Yes	[39]
CHB-8	Day 5 PQE	46,XX	Yes	NR	Yes	Yes	[39]
CHB-9	Day 5 PQE	46,XY	Yes	NR	Yes	Yes	[39]
CHB-10	Day 5 PQE	46,XY	Yes	NR	Yes	Yes	[39]
CHB-11	Day 5 PQE	46,XX	Yes	NR	Yes	Yes	[39]
CHB-12	Day 5 PQE	46,XX	Yes	NR	Yes	Yes	[39]
CU1	Day 6 arrested poor blastocyst	46,XX	Yes	Yes	ND	ND	[41]
CU2	Day 6 arrested early blastocyst	46,XX[a]	Yes	Yes	ND	ND	[41]

ND, not determined; *NR*, not reported; *PQE*, poor-quality embryo.
[a]*Putative normal karyotype: possible low level of mosaicism.*

CONCLUSION

The derivation of hESC from organismically dead embryos is a unique approach because it defines a common ground in the human ES debate. Harvesting live cells from dead human embryos has the likelihood of being accepted by the staunchest opponents of embryo-destructive ES derivation. ES cells generated by this approach appear to be suitable for clinical research. Thus far, 11 human ES lines derived by Daley and colleagues have been included in the NIH stem cell registry and are available for research with NIH funding [10]. Human ES lines generated from organismically dead embryos are of equal quality compared with lines derived by the classical, intracellular mass–derived approach, but further characterization of these lines is needed [2].

During routine IVF procedures, large proportions of embryos fail to develop properly [45,47,48] and are discarded as being unsuitable for clinical use [2,27]. Despite the low efficiency of isolation of hESC from organismically dead embryos, large-scale derivation is not limited because in the United States alone, nearly half a million such embryos are generated yearly as a by-product of assisted reproductive technologies [2,27]. The prospect of thousands of hESC lines generated by this method and deposited into stem cell banks renders clinical applications based on human leukocyte antigen matching feasible.

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CHAPTER

9

Stem Cells From the Amnion

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INTRODUCTION

In this chapter, we provide an overview of the potential advantages and disadvantages of different stem and progenitor cell populations identified in the amnion and in the amniotic fluid (AF), along with their properties and potential clinical applications.

Placenta, fetal membranes (i.e., amnion and chorion), and AF have been extensively investigated as a potential noncontroversial source of stem cells. They are usually discarded after delivery and are accessible during pregnancy through amniocentesis and chorionic villus sampling [1]. Several populations of cells with multilineage differentiation potential and immunomodulatory properties have been isolated from the human placenta and fetal membranes; they have been classified by an international workshop [2] as human amniotic epithelial cells (hAECs) [3–7], human amniotic mesenchymal stromal cells (hAMSCs) [8,9], human chorionic mesenchymal stromal cells (hCMSCs) [10,11], and human chorionic trophoblastic cells (hCTCs).

In the AF, two main populations of stem cells have been isolated: amniotic fluid mesenchymal stem cells (AFMSCs) and amniotic fluid stem (AFS) cells. Because of the easier accessibility of the AF compared with other extraembryonic tissues, these cells may hold much promise in regenerative medicine.

PLACENTA: FUNCTION, ORIGIN, AND COMPOSITION

During human placental development, a range of cell types is generated, depending on gestation, which can be described as epithelial (because they derive from the amniotic membrane), trophoblastic, and hematopoietic (both derived from the chorionic villi). Placental tissue has contributions from both the fetus (amniotic membrane epithelium, extraembryonic mesoderm, and the two-layered trophoblast) and the mother (decidua basalis). The primitive formation of the placenta occurs from cells of fetal origin, known as trophoblast, that invade the uterine endometrium, form the outer layer of the blastocyst, and produce a network of protrusions, the villi and the lacunae system. On the 7th to 10th day after conception, the chorionic membranes are developed from layers of proliferating placental cells. At day 9 postconception, the inner cell mass induces the formation of the epiblast and hypoblast that subsequently become the amniotic cavity and the yolk sac. The process of gastrulation enables the bilaminar disc to differentiate into the three germ layers (ectoderm, mesoderm, and endoderm), followed by organogenesis [12,13].

During the maturation of the syncytium, the villi establish the maternofetal transport of blood nutrients, oxygen, gases, and waste products, and they differentiate from mesenchymal villi into immature intermediate villi. The placental progenitor stem cells are the cytotrophoblast cells emanating from the trophectodermal layer that result in the villous syncytiotrophoblast, which is a multinucleated aggregate of cytotrophoblast cells, and the extravillous cytotrophoblasts (EVTs) [14]. The trophoblast invasion of the maternal decidualized endometrium is also associated with hormonal secretions, such as human chorionic gonadotrophin, which downregulates maternal cellular immunity and promotes angiogenic activity of the EVTs [15]. By the 12th week of gestation, the placenta has adopted a

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hemotrophic nutritional function, in particular causing extensive transformation of the maternal spiral arteries into augmented high-flow, low-resistance vessels that provide sufficient nutrients and oxygen for the developing fetus.

Human fetal placental cells can be divided into the hAECs, hAMSCs, hCMSCs, and hCTCs. Isolation of these latter cells (hCMSCs and hCTCs) can be realized directly from the placenta or through chorionic villus sampling, a form of invasive ultrasound-guided prenatal diagnosis that entails sampling of the placental tissue from 11 weeks of gestation. Isolation of the heterogeneous population of cells (hAECs, hCMSCs, and hCTCs) can be implemented from different placenta regions through enzymatic digestion with dispase or collagenase in synergy with DNase.

AMNIOTIC FLUID: FUNCTION, ORIGIN, AND COMPOSITION

The AF is the clear, watery liquid that surrounds the growing fetus within the amniotic cavity. It allows the fetus to grow freely and move inside the uterus, protects it from outside injuries by cushioning sudden blows or movements and by maintaining consistent pressure and temperature, and acts as a vehicle for the exchange of body chemicals with the mother [16,17].

In humans, the AF starts to appear at the beginning of the second week of gestation as a small film of liquid between the cells of the epiblast. Between days 8 and 10 after fertilization, this fluid gradually expands and separates the epiblast (i.e., the future embryo) from the amnioblasts (i.e., the future amnion), thus forming the amniotic cavity [6]. Thereafter, it progressively increases in volume, completely surrounding the embryo after the fourth week of pregnancy. Over the course of gestation, AF volume markedly changes from 20 mL in the seventh week to 600 mL in the 25th week, 1000 mL in the 34th week, and 800 mL at birth. During the first half of gestation, the AF results from active sodium and chloride transport across the amniotic membrane and the nonkeratinized fetal skin, with concomitant passive movement of water. In the second half of gestation, the AF is composed of fetal urine, gastrointestinal excretions, respiratory secretions, and substances exchanged through the sac membranes [18–21].

The AF is primarily composed of water and electrolytes (98%–99%) but it also contains chemical substances (e.g., glucose, lipids, proteins, hormones, and enzymes), suspended materials (e.g., vernix caseosa, lanugo hair, and meconium), and cells. AF cells are derived both from extraembryonic structures (i.e., placenta and fetal membranes) and from embryonic and fetal tissues [22]. Although AF cells are known to express markers of all three germ layers [23], their exact origin still represents a matter of discussion; the consensus is that they consist mainly of cells shed in the amniotic cavity from the developing skin, respiratory apparatus, and urinary and gastrointestinal tracts [18,24]. AF cells display a broad range of morphologies and behaviors varying with gestational age and fetal development [25]. Under normal conditions, the number of AF cells increases with advancing gestation; if a fetal disease is present, AF cell counts can be either dramatically reduced (e.g., intrauterine death, urogenital atresia) or abnormally elevated (e.g., anencephaly, spina bifida, exomphalos) [26]. Based on their morphological and growth characteristics, viable adherent cells from the AF are classified into three main groups: epithelioid (33.7%), AF (60.8%), and fibroblastic type (5.5%) [25]. In the event of fetal abnormalities, other types of cells can be found in the AF, e.g., neural cells in the presence of neural tube defects and peritoneal cells in case of abdominal wall malformations [26–28].

Most cells present in the AF are terminally differentiated and have limited proliferative capabilities [26,29]. In the 1990s, however, two groups demonstrated the presence in the AF of small subsets of cells harboring a proliferation and differentiation potential. First, Torricelli reported the presence of hematopoietic progenitors in the AF collected before the 12th week of gestation [30]. Then Streubel was able to differentiate AF cells into myocytes, which suggested the presence in the AF of nonhematopoietic precursors [31]. These results initiated new interest in the AF as an alternative source of cells for therapeutic applications.

AMNIOTIC EPITHELIAL CELLS

Human amnion consists of amniotic epithelial cells (AECs) on a basement collagenous membrane, an acellular compact layer filled with reticular fibers, a fibroblast layer with Hofbauer cells/histiocytes, and a highly hygroscopic spongy layer with fibrils between the chorion and the amniotic sac [32].

AECs can be obtained with differential enzymatic digestion from the amnion membrane after it is separated from the underlying chorion [33]. The amnion contains epithelial cells expressing surface markers that include both embryonic-specific markers such as the stage-specific antigens (SSEAs) 3 and 5, Tra-1-60, Tra-1-81, and mesenchymal markers CD105, CD90, CD73, CD44, CD29, human leukocyte antigen (HLA)-A, -B, -C, CD13, CD10, CD166, and

CD117. Their immunological properties have not been completely elucidated; however, AECs also appear to be resistant to rejection after allotransplantation, probably owing to their immunosuppression properties (CD59 and HLA-G [34]), which could also lead to their therapeutic role in a disease model. For example, hAECs have been reported to lower the blood glucose levels of streptozotocin-induced diabetic mice several weeks after implantation, potentially by differentiation into β cells [35].

hAECs are considered multipotent cells because of their capacity to differentiate into different lineages. In particular, they have been able to mature under specific culture conditions into neuronal cells that synthesize acetylcholine, norepinephrine, and dopamine [6,36,37]. In vivo, hAECs have been reported to be neuroprotective and neuroregenerative, probably in relation to growth factor secretion. Indeed, studies showed that hAEC-conditioned media exhibit neurotrophic effects on rat cortical cells [38], and because of the expression of neural markers such as nestin, glial fibrillary acidic protein, and microtubule-associated protein 2, they are inclined toward neuronal lineages. hAECs have also been used to treat peripheral nerve injuries in animal models, in which they have shown to enhance the growth of host neurons and guide regenerative sprouting [68a]. In addition to their neurogenic potential, some relevant work has been done on exploring the hepatic potential of AECs. First, AECs produce albumin and α-fetoprotein, and they show glycogen storage and hepatic differentiation potential in vitro [39,40]. Moreover, in vitro, hAECs had the capacity to metabolize ammonia, testosterone and 17α-hydroxyprogesterone caproate, whereas they expressed hepatocyte markers such as albumin, A1AT, CYP2A4, 3A7, 1A2, 2B6, ASGPR1, and inducible fetal cytochromes. After intrahepatic transplantation into immunodeficient (severe combined immunodeficient [SCID])/beige mice, hAECs demonstrated functional hepatic characteristics [39], and after pretreatment of SCID/beige mice with retrorsine, hAECs expressed mature liver genes, plasma proteins, and hepatic enzymes to a level equal to adult liver tissue. hAECs showed therapeutic efficacy after transplantation in a mouse model of cirrhosis [41]. The transplantation of human amnion−derived epithelial cells to the liver appears to have desired therapeutic properties including the secretion of matrix metalloproteinase that instigate fibrinolysis and increase in interleukin-10 concentration. In a liver disease mouse model, amnion epithelial cell transplantation resulted in hepatic engraftment with decreased inflammation, fibrosis, and hepatocyte apoptosis [42]. Zhang et al. infused amnion-derived cells in a carbon tetrachloride −-treated mouse liver, and they showed minimal fibrosis and apoptosis [43]. Ricci et al. used a piece of human amniotic membrane (hAM) to assess fibrosis in rat liver and demonstrated increased antifibrotic properties of the hAM with a reduction in ductular reaction and extracellular matrix (ECM) deposition [44]. Vaghjiani et al. showed that the differentiation of hAECs into hepatic-like cells can remain viable and functional after encapsulation in barium alginate microspheres in vitro, and they can express CYP3A4, which is thought to break down nearly 50% of all therapeutic drugs [45].

Moreover, the cryopreserved amniotic membrane and its by-products have been recognized as significant tools for the treatment of ulceration and epithelial defects (corneal or conjunctival) [46,47]. Nakamura et al. used autologous serum corneal epithelial cells on an amniotic membrane to transplant nine eyes of nine patients with total limbal stem cell deficiency, and they demonstrated improvement in visual acuity and complete corneal epithelialization within 2−5 days [48]. Wang et al. experimented on allogeneic green fluorescent protein $(GFP)^+$ mice intact amniotic epithelium grafts with syngeneic (EGFP-C57BL/6 to C57BL/6 W/t) and allogeneic (EGFP-C57BL/6 to BALB/c W/t) AE cells that were transplanted into the cornea or conjunctiva or inserted into the anterior chambers. The researchers showed that major histocompatibility complex (MHC) class I beta antigens were minimally expressed after implantation [49]. Finally, other potential clinical applications have been explored and AECs have been considered potentially useful for a broad variety of conditions including ophthalmic diseases, lung fibrosis, liver fibrosis, multiple sclerosis, congenital metabolic disorders such as ornithine transcarbamylase deficiency, familial hypercholesterolemia, spinal cord injuries, and Parkinson disease and for allogeneic cell transplantations [50−52].

AMNIOTIC MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs) represent a population of multipotent stem cells able to differentiate toward mesoderm-derived lineages (i.e., adipogenic, chondrogenic, myogenic, and osteogenic) [53]. Initially identified in adult bone marrow, where they represent 0.001%−0.01% of total nucleated cells [54], MSCs have since been isolated from several adult (e.g., adipose tissue, skeletal muscle, liver, and brain), fetal (i.e., bone marrow, liver, and blood), and extraembryonic tissues (i.e., placenta and amnion) [55].

The presence of a subpopulation of AF cells with mesenchymal features able to proliferate in vitro more rapidly than comparable fetal and adult cells was described for the first time in 2001 [56]. In 2003, In 't Anker demonstrated that the AF can be an abundant source of fetal cells that exhibit a phenotype and a multilineage differentiation

potential similar to that of bone marrow–derived MSCs (BM-MSCs); these cells were named AFMSCs [57]. Soon after that article, other groups independently confirmed similar results.

Isolation and Culture

AFMSCs can easily be obtained: in humans, from small volumes (2–5 mL) of second- and third-trimester AF [57a,61], where their percentage is estimated to be 0.9%–1.5% of the total AF cells [58]; and in rodents, from the AF collected during the second or third week of pregnancy [59,60]. Various protocols have been proposed for their isolation; all are based on the expansion of unselected populations of AF cells in serum-rich conditions without feeder layers, allowing cell selection by culture conditions. The success rate of the isolation of AFMSCs was reported by different authors to be 100% [61]. AFMSCs grow in basic medium containing fetal bovine serum (20%) and fibroblast growth factor (5 ng/mL). It was shown that human AFMSCs can be also cultured in the absence of animal serum without losing their properties [62]; this finding is a fundamental prerequisite for the beginning of clinical trials in humans.

Characterization

The fetal versus maternal origin of AFMSCs has been investigated by different authors. Molecular HLA typing and amplification of the SRY gene in AF samples collected from male fetuses [57,58] demonstrated the exclusive fetal derivation of these cells. However, whether AFMSCs originate from the fetus or from the fetal portion of extraembryonic tissues remains a matter of debate [62].

AFMSCs display a uniform spindle-shaped, fibroblast-like morphology similar to that of other MSCs populations and expand rapidly in culture [63]. Human cells derived from a single 2-mL AF sample can increase to 180×10^6 cells within 4 weeks (three passages), and as demonstrated by growth kinetics assays, they possess a greater proliferative potential (average doubling time, 25–38 h) compared with that of BM-MSCs (average doubling time, 30–90 h) [57,58,60,64]. Moreover, AFMSC clonogenic potential has been proved to exceed that of MSCs isolated from bone marrow (86 ± 4.3 versus 70 ± 5.1 colonies) [60]. Despite their high proliferation rate, AFMSCs retain a normal karyotype and do not display tumorigenic potential even after extensive expansion in culture [58,64].

Analysis of AFMSC transcriptome demonstrated that: (1) the AFMSC gene expression profile, as well as that of other MSC populations, remains stable between passages in culture, enduring cryopreservation and thawing well; (2) AFMSCs share with MSCs derived from other sources a core set of genes involved in ECM remodeling, cytoskeletal organization, chemokine regulation, plasmin activation, transforming growth factor-β, and Wnt signaling pathways; and (3) compared with other MSCs, AFMSCs show a unique gene expression signature that consists of the upregulation of genes involved in signal transduction pathways (e.g., HHAT, F2R, and F2RL) and in uterine maturation and contraction (e.g., OXTR and PLA2G10), which suggests a role of AFMSCs in modulating interactions between the fetus and the uterus during pregnancy [63].

Different investigators determined the cell surface antigenic profile of human AFMSCs through flow cytometry (Table 9.1). Cultured human AFMSCs are positive for mesenchymal markers (i.e., CD90, CD73, CD105, and CD166), for several adhesion molecules (i.e., CD29, CD44, CD49e, and CD54), and for antigens belonging to MHC-I. They are negative for hematopoietic and endothelial markers (e.g., CD45, CD34, CD14, CD133, and CD31).

AFMSCs exhibit a broad differentiation potential toward mesenchymal lineages. Under specific in vitro inducing conditions, they are able to differentiate toward the adipogenic, osteogenic, and chondrogenic lineage [57,60,63].

Despite not being pluripotent, AFMSCs can be efficiently reprogrammed into pluripotent stem cells (iPS) via retroviral transduction of defined transcription factors (Oct4, Sox2, Klf-4, and c-Myc). Strikingly, AFMSC reprogramming capacity is significantly higher (100-fold) and much quicker (6 days versus 16–30 days) compared with that of somatic cells such as skin fibroblasts. Similarly to iPS derived from other sources, iPS derived from AF cells generate embryoid bodies (EBs) and differentiate toward all three germ layers in vitro; in vivo, they form teratomas when injected into SCID mice [65].

Preclinical Studies

After the identification of AFMSCs, various studies investigated their therapeutic potential in different experimental settings. Different groups demonstrated that AFMSCs are able not only to express cardiac and

TABLE 9.1 Immunophenotype of Culture-Expanded Second- and Third-Trimester Human Amniotic Fluid Mesenchymal Stromal Cells: Results by Different Groups

Markers	Antigen	CD No.	You [57a]	Roubelakis [58]	Tsai [61]	In 't Anker [57]
Mesenchymal	SH2, SH3, SH4	CD73	+	+	+	+
	Thy1	CD90	+	+	+	+
	Endoglin	CD105	+	+	+	+
	SB10/ALCAM	CD166	nt	+	nt	+
Endothelial and hematopoietic	Leukocyte common antigen	CD14	nt	−	nt	−
	gp105-120	CD34	nt	−	−	−
	Lipopolysaccharide-R	CD45	−	−	−	−
	Prominin-1	CD133	nt	−	nt	nt
Integrins	β1-integrin	CD29	+	+	+	nt
	β3-integrin	CD61	−	nt	nt	nt
	α4-integrin	CD49d	nt	−	nt	−
	α5-integrin	CD49e	nt	+	nt	+
	Lymphocyte function −associated-1	CD11a	nt	+	nt	−
Selectins	E-Selectin	CD62E	nt	+	nt	−
	P-selectin	CD62P	nt	+	nt	−
Immunoglobulin superfamily	Platelet endothelial cell adhesion molecule-1	CD31	−	+	−	−
	Intercellular adhesion molecule (ICAM)-1	CD54	nt	+	nt	+
	ICAM-3	CD50	nt	−	nt	−
	Vascular cell adhesion protein molecule-1	CD106	nt	+	nt	−
	Homing cell adhesion molecule-1	CD44	nt	+	+	+
Major histocompatibility complex	I (human leukocyte antigen [HLA]-ABC)	none	nt	+	+	+
	II (HLA-DR, DP, DQ)	none	nt	nt	−	−

nt, not tested.

endothelial-specific markers under specific culture conditions, but also to integrate into normal and ischemic cardiac tissue, where they differentiate into cardiomyocytes and endothelial cells [66−69]. In a rat model of bladder cryoinjury, AFMSCs show the ability to differentiate into smooth muscle and prevent the compensatory hypertrophy of surviving smooth muscle cells [59].

AFMSCs can be a suitable cell source for tissue engineering of congenital malformations. In an ovine model of diaphragmatic hernia, repair of the muscle deficit using grafts engineered with autologous mesenchymal amniocytes leads to better structural and functional results compared with equivalent fetal myoblast-based and acellular implants [62,70]. Engineered cartilaginous grafts have been derived from AFMSCs grown on biodegradable meshes in serum-free chondrogenic conditions for at least 12 weeks; these grafts have been successfully used to repair tracheal defects in fetal lambs when implanted in utero [62]. The surgical implantation of AFMSCs seeded on nanofibrous scaffolds and predifferentiated in vitro toward the osteogenic lineage into a leporine model of sternal defect led to complete bone repair in 2 months [71].

Intriguingly, studies suggested that AFMSCs can harbor trophic and protective effects in the central and peripheral nervous systems. Pan showed that AFMSCs facilitate peripheral nerve regeneration after injury and

hypothesized that this can be determined by cell secretion of neurotrophic factors [72–74]. After transplantation into the striatum, AFMSCs are capable of surviving and integrating in the rat adult brain and of migrating toward areas of ischemic damage [75]. Moreover, the intraventricular administration of AFMSCs in mice with focal cerebral ischemia-reperfusion (IR) injuries significantly reverses neurological deficits in treated animals [76].

Remarkably, it was also observed that AFMSCs present in vitro had an immunosuppressive effect similar to that of BM-MSCs [77]. After stimulation of peripheral blood mononuclear cells with anti-CD3, anti-CD28, or phytohemagglutinin, irradiated AFMSCs demonstrated a significant inhibition of T-cell proliferation with a dose-dependent kinetics [64].

AMNIOTIC FLUID STEM CELLS

The first suggestion that the AF may contain undifferentiated cells was based on the observation that AF-derived cells expressed skeletal muscle proteins when cultured in the supernatant of rhabdomyosarcoma cell lines [31]. Subsequently, AF-derived cells were shown to differentiate into osteocytes, adipocytes, and fibroblasts while having a cell marker profile comparable to MSCs [57]. Brivanlou and colleagues were the first to confirm that a subpopulation of AF-derived cells (approximately 0.5%–1% of total live cells) have stem cell potential, by demonstrating the expression of octamer transcription factor-4 (Oct-4) at the transcriptional and protein levels [78]. Remarkably, Karlmark et al. transfected human AF cells with the GFP gene under either the Oct-4 or the Rex-1 promoter and established that some AF cells were able to activate these promoter [79]. Subsequently, we and others used CD117 (c-Kit; type III tyrosine kinase receptor for stem cell factor with essential roles in gametogenesis, melanogenesis, and hematopoiesis) as a means to select the undifferentiated population from the AF [80,81]. These CD117-expressing cells are a heterogeneous population; they were isolated from small [80–83] and large animals [84] as well as humans [80,85] and are known as AFSC.

Isolation and Culture

The proportion of c-kit$^+$ cells in the AF varies over the course of gestation, roughly describing a Gaussian curve; they appear at very early time points in gestation (i.e., at 7 weeks of amenorrhea in humans and at embryonic day (E)9.5 in mice) and present a peak at midgestation equal to 90×10^4 cells/fetus at 20 weeks of pregnancy in humans and 10,000 cells/fetus at E12.5 in mice [81]. Human AFS cells (AFSC) can be derived either from small volumes (5 mL) of second-trimester AF (14–22 weeks of gestation) or from confluent backup amniocentesis cultures. Murine AFSC are obtainable from the AF collected during the second week of gestation (E11.5–14.5) [80,86–88]. AFSC isolation is based on a two-step protocol consisting of the prior immunological selection of c-kit–positive cells from the AF (approximately 1% of total AF cells) and in the subsequent expansion of these cells in culture [80,87,89–92]. Isolated AFSC can be expanded in feeder layer-free, serum-rich conditions without evidence of spontaneous differentiation in vitro. Cells are cultured in basic medium containing 15% of fetal bovine serum and Chang supplement [80,92].

Characterization

Karyotype analysis of human AFSC deriving from pregnancies in which the fetus was male revealed the fetal origin of these cells [80].

AFSC proliferate well during ex vivo expansion. When cultivated, they display a spectrum of morphologies ranging from a fibroblast-like to an oval-round shape (Fig. 1A). As demonstrated by different authors, AFSC possess great clonogenic potential [80,88]. Clonal AFSC lines expand rapidly in culture (doubling time, 36 h); more interesting, they maintain a constant telomere length (20 kilobase pairs) between early and late passages (Fig. 1B). Almost all clonal AFSC lines express markers of a pluripotent undifferentiated state: Oct4 and NANOG [80,88,89,92,93]. However, they have been proved not to form tumors when injected in SCID mice [80].

Different investigators determined the cell surface antigenic profile of AFSC through flow cytometry (Table 9.2). Cultured human AFSC are positive for embryonic stem (ES) cell (e.g., SSEA-4) and mesenchymal markers (e.g., CD73, CD90, and CD105), for several adhesion molecules (e.g., CD29 and CD44) and for antigens belonging to MHC-I. They are negative for hematopoietic and endothelial markers (e.g., CD14, CD34, CD45, CD133, and CD31), and for antigens belonging to MHC-II.

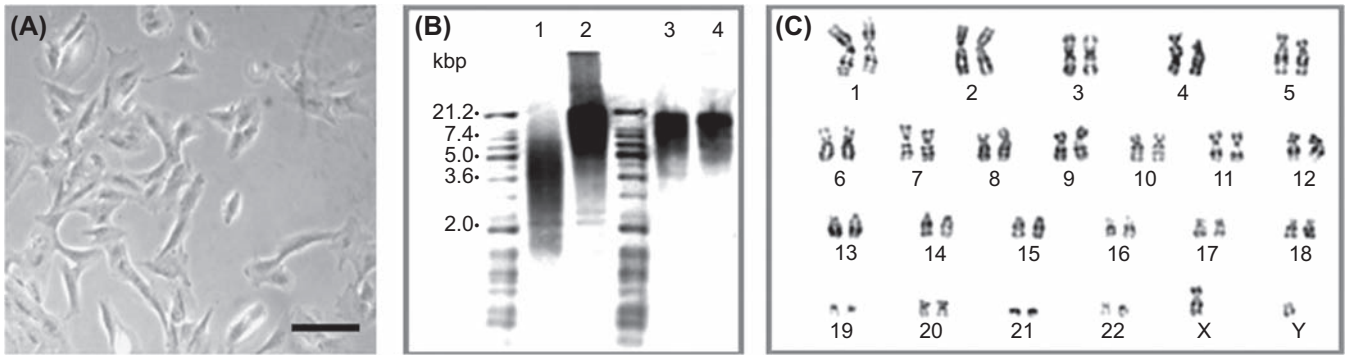

FIGURE 9.1 (A) Human amniotic fluid stem cells (AFSC) mainly display a spindle-shaped morphology during in vitro cultivation under feeder layer-free, serum-rich conditions. (B, C) Clonal human AFSC lines retain long telomeres and a normal karyotype after more than 250 cell divisions (magnification 20×). (B) Conserved telomere length of AFSC between early passage (20 population doublings, lane 3) and late passage (250 population doublings, lane 4). Short-length (lane 1) and high-length (lane 2) telomere standards provided in the assay kit. (C) Giemsa band karyogram showing chromosomes of late-passage (250 population doublings) cells. *Picture adapted from De Coppi (2007b).*

TABLE 9.2 Surface Markers Expressed by Human c-kit$^+$ Amniotic Fluid Stem Cells: Results by Different Groups

Markers	Antigen	CD No.	Ditadi [81]	De Coppi [80]	Kim [3]	Tsai [88]
Embryonic stem cells	Stage-specific antigen (SSEA)-3	None	nt	–	+	nt
	SSEA-4	None	nt	+	+	nt
	Tra-1-60	None	nt	–	+	nt
	Tra-1-81	None	nt	–	nt	nt
Mesenchymal	SH2, SH3, SH4	CD73	nt	+	nt	+
	Thy1	CD90	+	+	nt	+
	Endoglin	CD105	nt	+	nt	+
Endothelial and hematopoietic	Leukocyte common antigen	CD14	nt	nt	nt	–
	gp105-120	CD34	–	–	nt	–
	Lipopolysaccharide-R	CD45	+	–	nt	nt
	Prominin-1	CD133	–	–	nt	nt
Integrins	β1-integrin	CD29	nt	+	nt	+
Immunoglobulin superfamily	Platelet endothelial cell adhesion molecule-1	CD31	nt	nt	+	nt
	Intercellular adhesion molecule-1	CD54	nt	nt	+	nt
	Vascular cell adhesion protein molecule-1	CD106	nt	nt	+	nt
	Homing cell adhesion molecule-1	CD44	+	+	+	+
Major histocompatibility complex	I (human leukocyte antigen [HLA]-ABC)	None	+	+	+	+
	II (HLA-DR, DP, DQ)	None	–	–	–	–

nt, not tested.

Because the stability of cell lines is a fundamental prerequisite for basic and translational research, the capacity of AFSC to maintain their baseline characteristics over passages has been evaluated based on multiple parameters. Despite their high proliferation rate, AFSC and derived clonal lines show a homogeneous, diploid DNA content without evidence of chromosomal rearrangement even after expansion to 250 population doublings [80,89] (Fig. 1C). Moreover, AFSC maintain constant morphology, doubling time, apoptosis rate, cell cycle distribution,

and marker expression (e.g., Oct4, CD117, CD29, and CD44) up to 25 passages [89,92]. During in vitro expansion, however, cell volume tends to increase and significant fluctuations of proteins involved in different networks (i.e., signaling, antioxidant, proteasomal, cytoskeleton, connective tissue, and chaperone proteins) can be observed using a gel-based proteomic approach [89]; the significance of these modifications warrants further investigations but needs to be taken in consideration when interpreting experiments over several passages and comparing results from different groups.

AFSC and, more important, derived clonal cell lines are able to differentiate toward tissues representative of all three embryonic germ layers spontaneously, when cultured in suspension to form EBs, and when grown under specific differentiation conditions.

EBs consist of three-dimensional aggregates of ES cells, which recapitulate the first steps of early mammalian embryogenesis [93a–93c]. As ES cells, when cultured in suspension and without antidifferentiation factors, AFSC harbor the potential to form EBs with high efficiency: the incidence of EB formation (i.e., the percentage of EB recovered from 15 hanging drops) is estimated to be around 28% for AFSC lines and around 67% for AFSC clonal lines. Similar to ES cells, EB generation by AFSC is regulated by the mammalian target of rapamycin pathway and is accompanied by a decrease of Oct4 and Nodal expression and by an induction of endodermal (GATA4), mesodermal (Brachyury and HBE1) and ectodermal (Nestin and Pax6) markers [92,94].

Under specific mesenchymal differentiation conditions, AFSC express molecular markers of adipose, bone, muscle, and endothelial differentiated cells (e.g., lipoprotein lipase, desmin, osteocalcin, and vascular cell adhesion protein 1). In the adipogenic, chondrogenic, and osteogenic medium, AFSC respectively develop intracellular lipid droplets, secrete glycosaminoglycans, and produce mineralized calcium [86,88]. Under conditions inducing cell differentiation toward the hepatic lineage, AFSC express hepatocyte-specific transcripts (e.g., albumin, α-fetoprotein, and multidrug-resistant membrane transporter 1) and acquire the liver-specific function of urea secretion (Fig. 2A) [80]. Under neuronal conditions, AFSC are capable of entering the neuroectodermal lineage. After induction, they express neuronal markers (e.g., G protein–coupled inwardly rectifying K^+ potassium channels), exhibit barium-sensitive potassium current, and release glutamate after stimulation (Fig. 2B). Ongoing studies are investigating AFSC capacity to yield mature, functional neurons [95–97].

AFSC can be easily manipulated in vitro. They can be transduced with viral vectors more efficiently than can adult MSCs, and after infection, they maintain their antigenic profile and the ability to differentiate into different lineages [98]. AFSC labeled with superparamagnetic micrometer-sized iron oxide particles retain their potency and can be tracked noninvasively by magnetic resonance imaging (MRI) for at least 4 weeks after injection in vivo [99].

Preclinical Studies

Several reports have investigated the potential applications of AFSC in different settings.

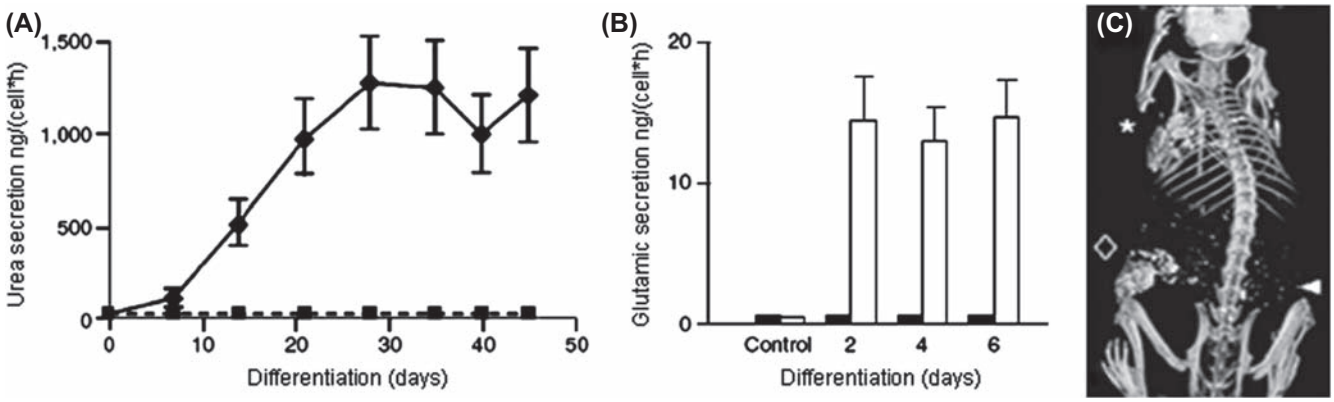

FIGURE 9.2 **Amniotic fluid stem cell (AFSC) differentiation into lineages representative of the three embryonic germ layers.** (A) Hepatogenic differentiation: urea secretion by human AFSC before (*rectangles*) and after (*diamonds*) hepatogenic in vitro differentiation. (B) Neurogenic differentiation: secretion of neurotransmitter glutamic acid in response to potassium ions. (C) Osteogenic differentiation: mouse micro-computed tomography scan 18 weeks after implantation of printed constructs of engineered bone from human AFSCs; *arrowhead*: region of implantation of control scaffold without AFSC; *rhombus*: scaffolds seeded with AFSC; *, bone deposit after transplantation. *Picture adapted from De Coppi (2007b).*

Musculoskeletal System

We investigated the osteogenic potential of AFSC by inducing osteogenic differentiation with culture media containing dexamethasone, β-glycerophosphate, and ascorbic acid-2-phosphate. After seeding in a collagen/alginate scaffold, they were implanted subcutaneously in immunodeficient mice. At 18 weeks, micro−computed tomography revealed highly mineralized tissues and blocks of bone-like material [80]. In a study by Sun et al., osteogenic differentiation of human AFSC was achieved using bone morphogenetic protein-7 and seeding on nanofibrous scaffolds, as evident by alkaline phosphatase activity, calcium content, von Kossa staining, and the expression of osteogenic genes. Implantation into the subcutaneous space led to bone formation in 8 weeks with positive von Kossa staining and a radio-opaque profile upon X-ray [100].

A series of experiments by the Goldberg laboratory investigated the osteogenesis of AFSC after seeding on a poly(ε-caprolactone) (PCL) biodegradable polymer. Cells that were differentiated in a three-dimensional PCL scaffold deposited mineralized matrix and were viable after 15 weeks of culture. It was also shown that predifferentiated cells in vitro produced seven times more mineralized matrix when implanted subcutaneously in vivo [101]. When the authors compared AFSC and BM-MSC for osteogenesis after seeding on scaffolds and long-term in vitro culture, they came across some striking results. Although BM-MSC differentiated more rapidly than AFSC, the growth and production of mineralized matrix halted at 5 weeks. In contrast, AFSC continued to produce matrix for up to 15 weeks, which led to a fivefold increase in overall production compared with BM-MSC seeded scaffolds.

We demonstrated for the first time the functional and stable long-term integration of AFSC into the skeletal muscle of human α-skeletal actin−Cre SmnF7/F7 mutant mice, which closely replicates the clinical features of human muscular dystrophy [102]. AFSC were obtained from E11.5−13.5 GFP^+ mice through direct CD117 selection immediately after AF collection. Approximately 25,000 freshly isolated AFSC were injected into the tail vein of each animal without previous expansion in culture. Transplanted mice displayed enhanced muscle strength, improved survival rate by 75%, and restored muscle phenotype compared with untreated animals. Not only was dystrophin distribution in GFP^+ myofibers similar to that of wild-type animals, GFP^+ cells were found to be engrafted into the muscle stem cell niche (as demonstrated by their sublaminal position and by Pax7 and α7-integrin expression). Functional integration of AFSC in the stem cell niche was confirmed by successful secondary transplants of GFP^+ satellite cells derived from AFSC-treated mice into untreated SmnF7/F7 mutant mice. To progress toward their application for therapy, the therapeutic potential of cultured AFSC was investigated and 25,000 AFSC, expanded under "embryonic-type" conditions, were intravenously injected into SmnF7/F7 mice. Cultured AFSC regenerated approximately 20% of the recipient muscle fibers compared with 50% when freshly isolated AFSC were used; this highlighted the importance of optimizing cell expansion protocols. Available data suggest that AFSC can differentiate toward myogenic lineages, engraft into the muscle stem cell niche, and participate in muscle regeneration in animal models of muscle injury. Hence, AFSC constitute a promising therapeutic option for skeletal muscle degenerative diseases.

Nervous System

We previously investigated human AFSC injection in the brain of Twitcher mice (a model of Krabbe globoid leukodystrophy associated with progressive oligodendrocyte and neuronal loss). Human AFSC engrafted into the lateral cerebral ventricles differentiated to cells that were similar to the surrounding environment and survived for up to 2 months. It was also demonstrated that engraftment was variable; 70% of AFSC survived in the brain of Twitcher mice, in contrast to only 30% of AFSC in the brain of normal mice [80]. A study by Prasongchean et al. indicated that treatment with small molecules, which normally leads to neuronal differentiation and grafting of AFSC into environments such as organotypic rat hippocampal cultures and the embryonic chick nervous system, led to no expression of neural cell markers. However, AFSC reduced hemorrhage and increased survival in a chick embryo model of extensive thoracic crush injury. Survival was not improved by mesenchymal or neural cells, or desmopressin. The authors explained that this effect was associated with the secretion of paracrine factors as evidenced by a transwell coculture model [103].

Heart

We previously looked at the cardiomyogenic potential of AFSC in vitro and in vivo. AFSC cultured in cardiomyocyte induction media or in coculture with cardiomyocytes demonstrated the expression of proteins specific for cardiomyocytes (atrial natriuretic peptide and α-myosin heavy chain), endothelial (CD31 and CD144), and smooth muscle cells (α-smooth muscle actin). In our first experience with xenogenic transplantation, human AFSC were transplanted in a rat model of myocardial infarction (MI). Cells of the immune system were recruited including

T, B, and NK cells and macrophages, and resulted in cell rejection. We speculated that this may be caused by AFSC expression of B7 costimulatory molecules CD80 and CD86 as well as macrophage marker CD68 [104]. In the next step, we attempted allogenic rat AFSC cardiac therapy by intracardiac transplantation in rats with IR injury. Three weeks after transplantation, a portion of the cells acquired an endothelial or smooth muscle phenotype and a smaller number had cardiomyocyte characteristics. Left ventricular ejection fraction was improved in the animals that had the AFSC injection, as quantified using MRI, and which suggested a paracrine therapeutic effect [82]. We then aimed to investigate this paracrine effect further using a rat model of MI and xenogeneic transplantation of human AFSC administered intravascularly immediately after reperfusion. This was dissimilar to our previous attempt with xenogeneic cellular cardiomyoplasty, which involved intramuscular injection of human AFSC within 20 min of coronary artery occlusion without reperfusion. Intravascular injection of human AFSC and their conditioned medium was cardioprotective and improved cell survival, and it decreased the infarct size from 54% to around 40% in both cases. We also showed that AFSC secrete the actin monomer-binding protein thymosin β-4 (Tβ-4), a paracrine factor with cardioprotective properties [105]. Tβ-4 has also been implicated as being cardioprotective in MI models that involved BM-MSC injection [106]. In addition to models of myocardial IR injury, we investigated the salutary effects of AFSC in a rat model of right heart failure resulting from pulmonary hypertension. After intravascular injection, AFSC engrafted in the lung, heart, and skeletal muscle reduced brain natriuretic peptide, a surrogate marker for heart failure, and proinflammatory cytokines. Moreover, AFSC differentiated into endothelial and vascular cells forming microvessels, capillaries, and small arteries. A 35% decrease in pulmonary arteriole thickness accompanied the injection [107]. Of relevance, in a seminal article, Rafii demonstrated that it was possible to convert human midgestation AF-derived cells directly into a stable and expandable population of vascular endothelial cells without using pluripotency factors [108]. Notably, it has been shown that ckit^{+} AFSC prior differentiation express early endothelial transcription factors. Moreover, in vivo, AFSC from both second and third trimesters expanded in hypoxia were able to rescue surface blood flow when locally injected in mice after chronic ischemia damage, and possessed the ability to fix carotid artery electric damage [109].

Hematopoietic System

Ditadi and colleagues were the first to demonstrate the hematopoietic potential of murine and human CD117^{+}/Lin^{-} AFSC [81]. In vitro, the AFSC population in both species displayed multilineage hematopoietic potential, as demonstrated by the generation of erythroid, myeloid, and lymphoid cells. In vivo, cells belonging to all hematopoietic lineages were found after primary and secondary transplantation of murine AFSC into immunocompromised hosts, thus demonstrating the long-term hematopoietic repopulating capacity of these cells. The latter results support the idea that the AF may be a source of stem cells with the potential for therapy of hematological disorders.

One of the most exciting applications of AFSC in this setting is in the field of in utero transplantation (IUT). IUT has been proposed as a novel approach for the treatment of inherited hematological disorders (including thalassemia and sickle cell disease) before birth [110]. Clinical translation has been limited by competitive and immunological barriers associated with IUT of adult bone marrow−derived HSC [111]. The use of AFSC for IUT could address many of these limitations. AFSC are of fetal origin, and as a result should be able to compete better against host cells compared with adult stem cells (potentially overcoming competitive barriers to engraftment). They are nonimmunogenic to the fetus at any gestational age and are also unlikely to result in maternal immunization because of the tolerogenic properties of the placenta. IUT of AFSC would involve harvesting the cells from the AF, employing in vitro gene therapy to correct the genetic defect, and transplanting back to the donor fetus. Such a combined autologous stem cell−gene transfer approach would also address some of the risks associated with administering gene therapy directly to the fetus (in utero gene therapy) [111]. The possibility of performing in vitro gene transfer to harvested ASFC would allow cells to be checked for insertional mutagenesis before transplantation and would obviate the risk of germline transmission of transgenes. In proof of principle studies, Shaw and colleagues showed that IUT of autologous (isolated using ultrasound-guided amniocentesis), expanded, and transduced AFSC resulted in widespread tissue engraftment (including the hematopoietic system) in the ovine fetus [112]. We are investigating the hematopoietic potential of freshly isolated and expanded AFSC after intravenous transplantation in immunocompetent fetal mice, and have obtained stable, multilineage engraftment at near-therapeutic levels using relatively small donor cell numbers. Whether in utero stem cell−gene therapy with AFSC would be therapeutic in models of hematological and other congenital disorders remains to be determined.

Kidney

AFSC have been shown to have plastic regenerative properties in the lung, by differentiating into different lineages according to the type of lung injury taking place in animal models of disease. AFSC injected intravascularly into

nude mice subjected to hyperoxia-induced pulmonary injury migrated to the lung and expressed human pulmonary epithelial differentiation marker thyroid transcription factor 1 and type-II pneumocyte marker surfactant protein C. After naphthalene injury to Clara cells, AFSC expressed the Clara cell−specific 10-kDa protein [113]. Moreover, in an adult rat model of hyperoxic lung injury, treatment with human AFSC has a reparative potential through paracrine involvement in alveolarization and angiogenesis [114]. In an established nitrofen-induced rat model of lung hypoplasia, lung growth, bronchial motility, and innervation were rescued by AFSC both in vitro and in vivo, which was similarly to results observed before with retinoic acid. The beneficial effect of AFSC was probably related to the paracrine action of growth factor secretion [115]. Those results have been validated in fetal rabbit with a surgically created left diaphragmatic hernia at day 23 (term, day 32). In this model, human AFSC exert an additional effect on tracheal occlusion leading to a decrease in mean terminal bronchiole density, a measure of alveolar number surrounding the terminal bronchioles, without signs of toxicity [116].

Lung

The first evidence regarding a nephrogenic potential of AFSC arose from a series of experiments involving the ex vivo growth of murine embryonic kidneys that were injected with labeled AFSC. Whereas AFSC were viable for up to 10 days' growth, they were also shown to contribute to a number of components of the developing kidney, such as the renal vesicle and S- and C-shape bodies. In addition, the ECM and surrounding cells induced renal differentiation, with the AFSC expressing kidney markers (zona occludens-1, glial-derived neurotrophic factor, and claudin) [91].

In a mouse model of acute tubular necrosis (ATN) involving glycerol injection, luciferase-labeled injected AFSC homed to the injured kidney. This decreased creatinine and blood urea nitrogen (BUN) levels and reduced the number of damaged tubules while increasing the proliferation of tubular epithelial cells. Interestingly, AFSC injected during the acute phase of ATN (between 48 and 72 h) had no effect on creatinine and BUN levels, whereas AFSC injected into the kidney on the same day of glycerol injection resulted in no observed peaks in creatinine or BUN. The authors speculated that this may be the result of AFSC accelerating the proliferation of partially damage epithelial tubular cells while preventing apoptosis [117]. Another laboratory confirmed the protective effect of AFSC in the same mouse model of ATN while comparing their effect with MSC. In addition to results regarding the amelioration of the effect of glycerol, it was demonstrated that MSC were more efficient in inducing proliferation and AFSC were more antiapoptotic. Sedrakyan et al. used Col4a5(−/−) mice as a mouse model of Alport syndrome to assess the effect of AFSC in renal fibrosis [83]. Early intracardiac administration of AFSC delayed interstitial fibrosis and the progression of glomerular sclerosis and prolonged animal survival. However, AFSC were not demonstrated to differentiate into podocytes, which suggests that the positive effects to the basement membrane were again mediated, as in other model of disease, by a paracrine mechanism [102,118]. It was reported for the first time that AFSCs mixed with organoids made with murine embryonic kidney contributed to the formation of glomerular structures, differentiated into podocytes with slit diaphragms, and internalized exogenously infused bovine serum albumin, attaining unprecedented (for donor stem cells) degrees of specialization and function in vivo [119].

Intestine

We looked at the effect of AFSC in a rat model of necrotizing enterocolitis (NEC) [120]. After 24 h of life, NEC rats were randomized to treatments of AFSC, BM-MSC, myoblasts (as a committed negative control), or phosphate-buffered saline (PBS) via intraperitoneal administration. NEC rats treated with AFSC showed significantly higher survival at 7 days compared with all the other groups and had an improved NEC clinical status at 96 h. MRI displayed significantly decreased peritoneal fluid accumulation (a surrogate marker for NEC grade) in the AFSC-treated rats. The improved clinical picture of the pups injected with AFSC was also evident by measurement of intestinal permeability, contraction, and motility.

The clinical data were confirmed by histological analysis, demonstrating a decreased amount of villus sloughing, core separation, and venous congestion. The relationship between these therapeutic effects and the presence of AFSC in the intestine was confirmed by tracking AFSC expressing GFP. At 48 h, cells were adherent to the mesentery; at 72 h, they were found in the serosa and muscularis; and at 96 h, they were located in the villi. The low cell numbers in these locations alongside the great clinical differences among treated groups suggested a paracrine effect. Accordingly, when we performed microarray analysis we saw differences in a number of genes involved in inflammation and tissue repair, cell cycle regulation, and enterocyte differentiation. These results were corroborated by immunofluorescence analysis examining cell proliferation and apoptosis.

We then sought to investigate the paracrine mechanism by which AFSC mediate their therapeutic effect and established that the number of cryptal cells expressing cyclo-oxygenase-2 ($COX\text{-}2^{+}$) inversely correlated with the

degree of intestinal damage. COX-2^+ cells were diminished in rats treated with PBS, whereas they were maintained in rats treated with AFSC. Interestingly, although the total number of COX-2^+ cells in villi was similar in AFSC-treated and control animals, cryptal COX-2^+ cells were significantly increased in the AFSC rats compared with both control animals and pups treated with PBS. This dependence on COX-2 was confirmed when the effect of AFSC was abolished both by selective COX-2 and nonselective COX inhibition, but it remained unaffected by a selective COX-1 inhibitor [121].

CONCLUSIONS

Many stem cell populations (e.g., embryonic, adult, and fetal stem cells) as well as methods for generating pluripotent cells (e.g., nuclear reprogramming) have been described. All of them have specific advantages and disadvantages. It has yet to be established which type of stem cell represents the best candidate for cell therapy. However, although it is likely that one cell type may be better than another, depending on the clinical scenario, the discovery of easily accessible cells of fetal derivation, not burdened by ethical concerns, in the AF has the potential of expanding new horizons in regenerative medicine. In fact, amniocentesis is routinely performed for the antenatal diagnosis of genetic diseases and its safety has been established by several studies documenting an extremely low overall fetal loss rate (0.06%−0.83%) related to the procedure [122,123]. Moreover, stem cells can be obtained from AF samples without interfering with diagnostic procedures.

Two stem cell populations have been isolated from the AF (i.e., AFMSCs and AFSC) and both can be used as primary (not transformed or immortalized) cells without further technical manipulations. AFMSCs exhibit typical MSC characteristics: fibroblastic-like morphology, clonogenic capacity, multilineage differentiation potential, immunosuppressive properties, expression of a mesenchymal gene expression profile, and a mesenchymal set of surface antigens. However, ahead of other MSC sources, AFMSCs are easier to isolate and have better proliferation capacities. The harvest of bone marrow remains a highly invasive and painful procedure, and the number, proliferation, and differentiation potential of these cells decline with increasing age [124,125]. Similarly, umbilical cord blood−derived MSCs exist at a low percentage and expand slowly in culture [126].

AFSC, on the other hand, represent a class of broadly stem cells with intermediate characteristics between ES and AS cells [29,127]. They express both embryonic and MSC markers, are able to differentiate into lineages representative of all embryonic germ layers, and do not form tumors after implantation in vivo. However, AFSC have been identified only recently and many questions need to be answered concerning their origin, epigenetic state, immunological reactivity, regeneration, and differentiation potential in vivo. AFSC may not differentiate as promptly as do ES cells and their lack of tumorigenesis can be argued against their pluripotency.

Although further studies are needed to better understand their biological properties and define their therapeutic potential, stem cells present in the AF appear to be promising candidates for cell therapy and tissue engineering. In particular, they represent an attractive source for the treatment of perinatal disorders such as congenital malformations (e.g., congenital diaphragmatic hernia) and acquired neonatal diseases requiring tissue repair/regeneration (e.g., NEC). In a future clinical scenario, AF cells collected during a routinely performed amniocentesis could be banked, and in case of need, subsequently expanded in culture or engineered in acellular grafts [29,62]. In this way, affected children could benefit from having autologous expanded or engineered cells ready for implantation either before birth or in the neonatal period [128].

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CHAPTER

10

Cord Blood Stem Cells

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INTRODUCTION

Umbilical cord blood (CB) is firmly established as an unrelated donor source for hematopoietic stem cell transplantation (HSCT) and is a readily available cell source in the evolving fields of regenerative medicine and cellular therapies. Worldwide, there are over 160 public banks with a global inventory of over 700,000 fully characterized, high-quality cord blood units (CBUs) [1], and more than 5 million CBUs have been banked at an estimated 215 family banks. In this chapter, we review the history of CB transplantation (CBT) and banking as well as established and emerging clinical uses of CB in regenerative medicine.

A BRIEF HISTORY

CB was first recognized as rich source of hematopoietic stem and progenitor cells (HSCs) several decades ago. In a pivotal series of experiments, Dr. Ted Boyce, working with Dr. Hal Broxmeyer and colleagues, demonstrated that CB HSCs possessed high proliferative potential, could successfully repopulate hematopoiesis in murine models and tolerated cryopreservation and thawing with efficient HSC recovery [2]. This critical work provided the scientific rationale to investigate CB as a potential source of donor HSCs in humans.

The first patient to undergo a CBT was a 5-year-old boy with Fanconi anemia. Through prenatal testing, it was determined that his mother was pregnant with an unaffected human leukocyte antigen (HLA) matched sibling. Upon the sibling's birth, the CB was collected into a sterile bottle containing preservative-free heparin and transported to Dr. Broxmeyer's laboratory, where it was diluted with tissue culture media and dimethyl sulfoxide (DMSO). The CB was then cryopreserved and stored under liquid nitrogen until it was transported to Paris, France, where the transplant would occur. The clinicians caring for the family elected to wait until the healthy sibling donor was 6 months of age so that she could serve as a backup bone marrow donor if the cord blood transplant failed. In 1988, Dr. Eliane Gluckman performed the first CBT in the world using the sibling's CB as the donor [3]. The child successfully engrafted with his sister's cells and has remained healthy with full donor chimerism ever since. Building on this initial success, additional related donor CBTs were performed in selected centers over the next 5 years [4–7]; the first unrelated donor CB bank was established by Dr. Pablo Rubinstein at the New York Blood Center in 1992. In the following year, Dr. Joanne Kurtzberg performed the first unrelated donor CBT at Duke University in a 4-year-old child with relapsed T-cell leukemia. Early experience with unrelated CBT demonstrated that partially HLA-mismatched, banked unrelated donor CB could successfully restore hematopoiesis with an incidence of graft versus host disease (GvHD) lower than expected and that engraftment was associated with the total nucleated cell (TNC) dose available relative to the recipient's body size [8,9].

With the extension into the unrelated donor setting, the fields of CBT and banking expanded rapidly. In 1995, EUROCORD was established by Dr. Eliane Gluckman; it continues to operate as an international CBT registry on behalf of the European Group for Blood and Marrow Transplantation. In 1996, the Foundation for the Accreditation of Cellular Therapy (FACT) was established by its parent organizations, the International Society for Cellular Therapy and the American Society of Blood and Marrow Transplantation (ASBMT). The following year, the International NetCord Foundation was established to serve as a registry for international public banks. The first

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international public CB banking standards were created by NetCord and the first accreditation program resulted from a collaboration between FACT and NetCord in 1999. Although participation is these programs is considered voluntary, many public CB banks are required to received accreditation from FACT/NetCord or the American Association of Blood Banks to participate in registries and receive reimbursement for units distributed for transplantation.

In the United States, the National Marrow Donor Program (NMDP) established the Center for Cord Blood in 1999 and expanded the unrelated donor registry to include publically banked CBUs. In 2006, after passage of the Stem Cell Act of 2005, Congress established the CW Bill Young Cell Transplantation Program. This program awarded contracts to the NMDP to create a single point of access donor registry and coordinating centers for CB and adult donors, to the Center for International Blood and Marrow Transplant Research to develop a stem cell outcomes database, and to the Health Resources and Services Administration of the Department of Health and Human Services to administer these programs and establish the national cord blood inventory (NCBI), a US network of public CB banks. This program was reauthorized in 2010 and 2015. A major goal of the program is to create a large repository of high-quality CBUs from donors of diverse ancestry to enable access to HSCT for as many patients as possible. In the United States, the Food and Drug Administration (FDA) regulates unrelated donor CB as biological product and issued final guidance for public banks to obtain a Biological License Agreement (BLA) in 2011. Seven public banks in the United States have obtained a BLA.

CORD BLOOD BANKING

Historically, after the birth of a baby, the CB and placenta were discarded as medical waste. With the advent of CBT, methods to reliably collect, process, test, cryopreserve, and store CB were developed with the goal of creating banks that could provide ready access to safe CBU products suitable for transplantation. Since the establishment of the first unrelated donor CB bank, the banking industry exploded, with an estimated worldwide inventory of more than 6 million CBUs stored in a combination of public and family CB banks [10]. Although the "shelf life" of banked cord blood is not known, successful transplants have been performed with units stored as long as 25 years. As the field has grown and advanced, accrediting agencies have emerged and developed standards for quality banking practices. In the United States, the FDA regulates unrelated CB as a biological product.

PUBLIC VERSUS FAMILY (OR PRIVATE) BANKS

There are two main types of CB banks. Public banks collect, process, and store donated CBUs intended for unrelated allogeneic transplantation at no expense to the donor or their family. Mothers consent to donate their baby's CB for public use and thereby relinquish all future rights to the unit. In the United States and most other countries, public banks are subject to strict regulatory oversight and use stringent volume, cell count, sterility, donor eligibility, and medical history specifications to determine which collected CBUs are banked. Because cell count is a critical determinant of CBU use, only larger units containing sufficient cells for a single or double CBT of an adult-sized individual are banked. As a result, many donated units do not qualify to be banked and are discarded or used for research, depending on the consent given by the donor family.

Family (or private) banks are generally "for-profit" businesses that charge the parents an initial processing fee and an annual storage fee to store CB exclusively for use by the child or the family. In actuality, the likelihood of using a privately banked CB for transplantation is low [11]. Therefore, the American Academy of Pediatrics, American Congress of Obstetricians and Gynecologists (ACOG), and ASBMT and other similar organizations worldwide do not recommend banking CB for personal use unless there is a family history of a disease (e.g., malignancy or hemoglobinopathy) that is amenable to HSCT [11–13]. To facilitate banking in these instances, many public and family banks offer "directed donor" programs, some of which waive charges associated with the banking process. Family banks are not subjected to the same regulatory oversight as public banks, although this varies among countries. Family banks generally use less stringent criteria for banking, which leads to wide variations in volume and cell content of the private inventory. According to a study of the safety of autologous CB infusions to treat children with acquired brain injury, CBUs from family banks were inferior to those stored in public banks with respect to collection volume, total nucleated cell count (TNCC), and $CD34^+$ count (Fig. 10.1) [14]. Because the use of CB is likely to expand, particularly in the field of regenerative medicine, the

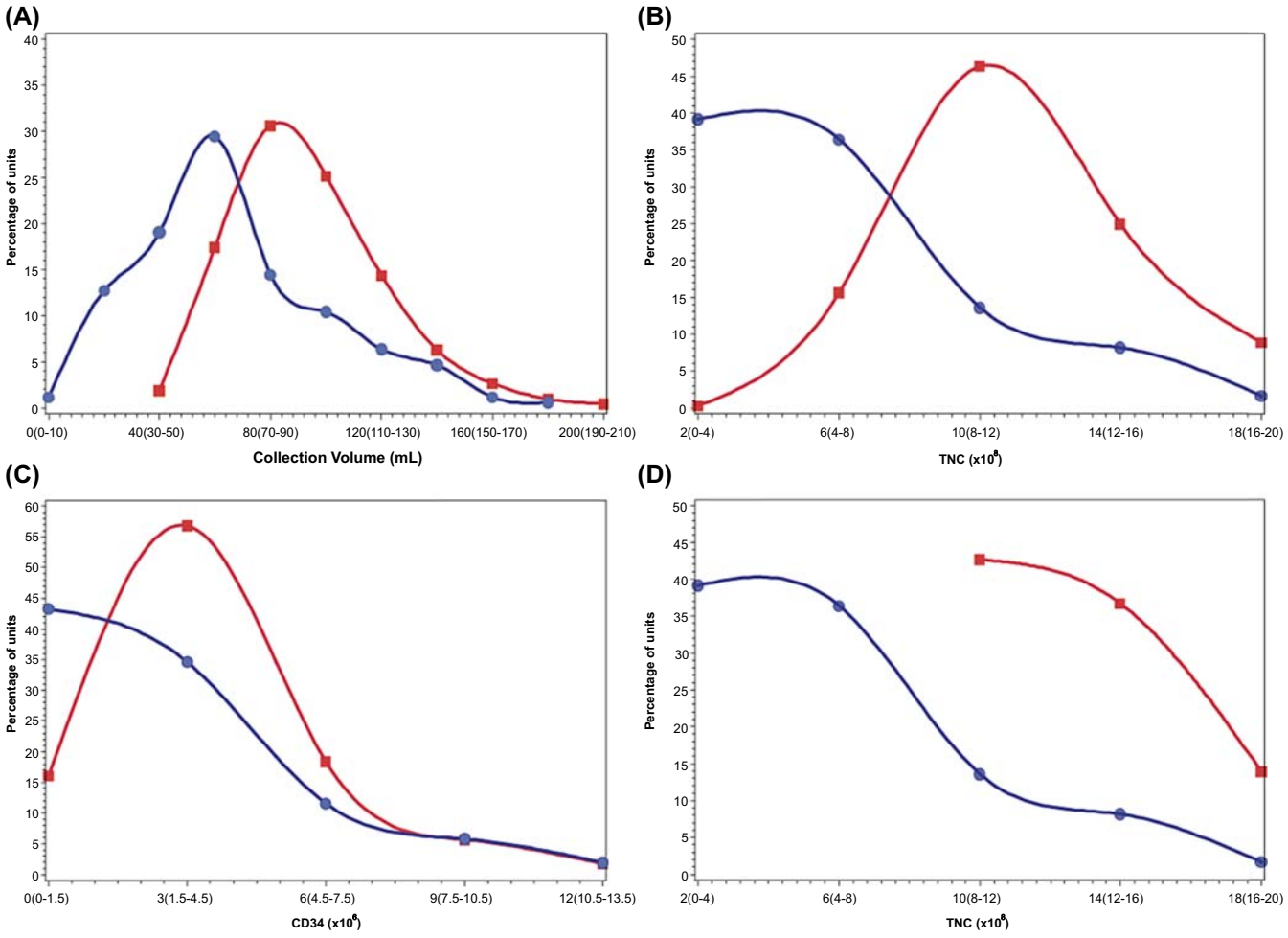

FIGURE 10.1 **Distributions of quality variables.** In (A–C), the distribution of autologous CBUs is compared with the entire Carolinas Cord Blood inventory with respect to collection volume (A), TNC (B), and CD34 content (C). In (D), TNC of autologous CBUs (represented as *red circles*) and National Cord Blood Inventory–eligible Carolinas Cord Blood Bank CBUs (represented as *blue squares*) are compared. *CBUs*, cord blood units; *TNC*, total nucleated cell. *Used with permission from Sun J, Allison J, McLaughlin C, et al. Differences in quality between privately and publicly banked umbilical cord blood units: a pilot study of autologous cord blood infusion in children with acquired neurologic disorders. Transfusion 2010;50(9):1980–7.*

indications and criteria that a CBU must meet for use may change. In response to these changes, the role of regulatory oversight in family CB banking may need to be optimized.

PUBLIC CORD BLOOD BANKING PROCEDURES

Donor Recruitment and Consent

Mothers who are potentially eligible for donation are identified based on clinical characteristics defined by the individual bank. At the Carolinas Cord Blood Bank (CCBB), donations are accepted from healthy mothers (aged ≥18 years) who are carrying healthy singleton pregnancies of at least 34 weeks' gestation and who provide written informed consent for donation before collection. Consent gives permission to collect the CBU. For potentially eligible units, the mother is approached again to give written informed consent for banking the unit for use in transplantation. These mothers provide a medical and family history, a maternal blood specimen to screen for certain communicable diseases, and a review of medical records of the infant and maternal donors. The mother also gives permission for the CB to be used for research if it does not meet specifications for banking.

Collection Techniques

CB can be collected from either vaginal or cesarean births, either before delivery of the placenta (in utero) by obstetrical staff or after delivery of the placenta (ex utero), allowing for dedicated, trained bank staff to perform collections. Although the delivery method is dictated by the clinical status of the mother and infant, the method of collection is determined by staffing and collection site practices. Generally, reports have observed higher collection volumes after cesarean compared with vaginal deliveries [15–17] and when CB is collected in utero compared with ex utero [18], although not all reports have agreed [19]. Both collection methods continue to be used routinely, but in utero collections are more common, likely owing to the additional personnel expenses associated with ex utero collections. Regardless of the collection method, CB is typically collected by cannulating the umbilical vein, allowing the placental blood to drain by gravity into collection containers with anticoagulant, most commonly citrate phosphate dextrose. Closed system collection bags are standard, because they have been shown to reduce bacterial contamination [20].

Volume and Cell Count Considerations

The goal of public CB banks is to provide quality CBUs for allogeneic transplantation for all patients in need. Because successful CBT requires a minimum TNC per kilogram dose, thresholds for banking have been established based on the TNCC. It is well-established that collection volume and TNCC are closely correlated. Therefore, many banks weigh the CBU after collection to estimate the volume and then ship to the processing laboratory only units that surpass a specified volume threshold. Low-volume units are discarded at the collection site because they are unlikely to have sufficient TNCC. Alternatively, some banks measure TNCC at the collection site or use other criteria to determine which units proceed to processing.

Efforts to increase collection volume have focused on two general approaches: identifying donations likely to have higher collection volume and developing techniques to obtain the maximal volume from an individual donation. Increased donor birth weight and older gestational age have been closely associated with higher collection volume and TNCC [21–23], although data suggest that collections from younger infants (aged 34–37 weeks' gestation) are more likely to have higher progenitor cell content, as measured by $CD34^+$ and colony-forming unit (CFU) content (Fig. 10.2) [24]. Also, despite comparable collection volumes among donors of different races or ethnicities, the TNCC, $CD34^+$, and CFU content adjusted for collection volume (counts/mL) are lower in individuals of certain racial backgrounds, particularly African Americans, compared with Caucasian donors, even after adjusting for other

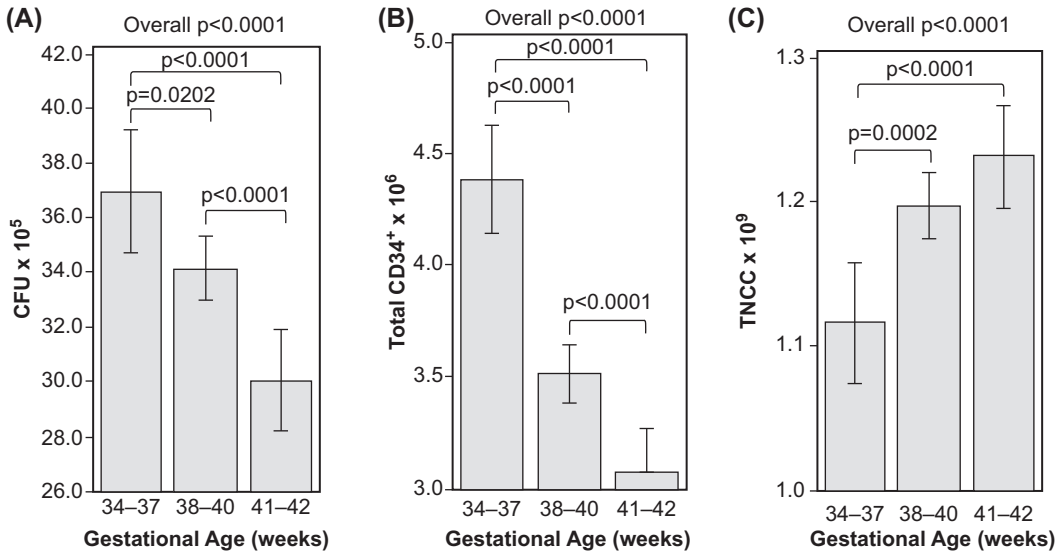

FIGURE 10.2 Impact of infant estimated gestational age on the CFU, $CD34^+$, and post-TNCC content. In (A–C), the adjusted mean CFU (A), $CD34^+$ (B), and post-TNCC (C) is shown in relationship to infant gestational age after adjusting for infant race/ethnicity, birth weight, sex, collection volume, delivery type, and maternal age. Only significant *P* values are shown. Whisker plots represent the 95% confidence intervals. *CFU*, colony-forming unit; *TNCC*, total nucleated cell count. *Used with permission from Page KM, Mendizabal A, Betz-Stablein B, et al. Optimizing donor selection for public cord blood banking: influence of maternal, infant, and collection characteristics on cord blood unit quality. Transfusion 2013;54.*

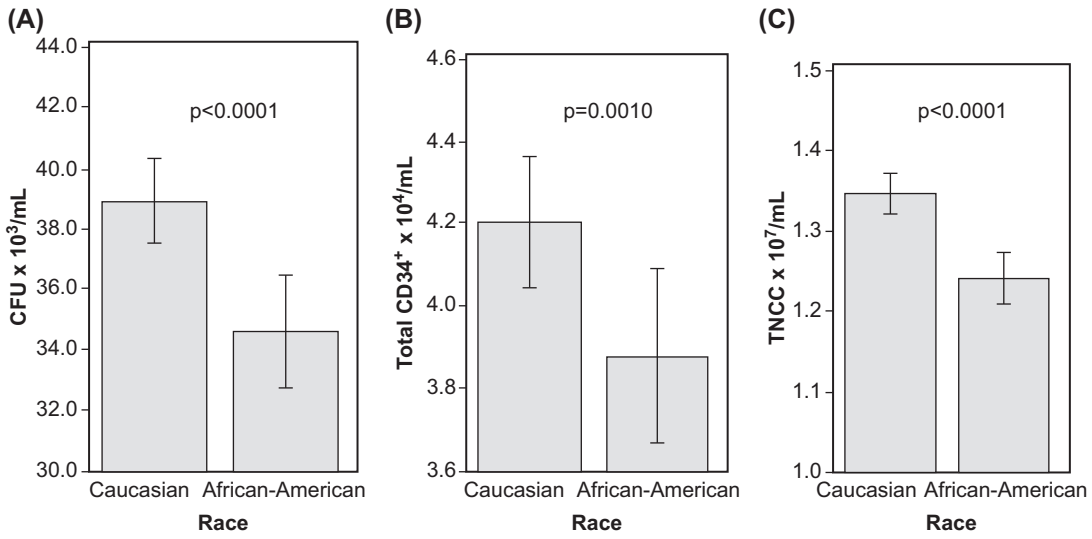

FIGURE 10.3 Comparison of the CFU, CD34$^+$, and post-TNCC concentrations for Caucasian and African American infants. In (A–C), the adjusted mean CFUs/mL (A), CD34$^+$ per mL (B), and post-TNCC/mL (C) are shown in relationship to race for infants of Caucasian and African American race, respectively, after adjusting for infant gestational age, birth weight, sex, collection volume, delivery type, and maternal age. Only significant *P* values are shown. Whisker plots represent the 95% confidence intervals. *CFU*, colony-forming unit; *TNCC*, total nucleated cell count. *Used with permission from Page KM, Mendizabal A, Betz-Stablein B, et al. Optimizing donor selection for public cord blood banking: influence of maternal, infant, and collection characteristics on cord blood unit quality. Transfusion 2013;54.*

clinical factors (Fig. 10.3) [15,24]. Investigations of other clinical factors such as gender and maternal age have been less conclusive [16,24–26]. Although these clinical characteristics are not typically modifiable, understanding their relationship to collection volume and content can inform practical decisions pertaining to unit eligibility, staffing at collection sites, and other banking practices.

Technical approaches to increasing CBU collection volume include increasing perfusion of the placenta to collect additional blood [27,28], but these approaches remain experimental. The timing of cord clamping also affects the volume of blood collected from a placenta. The practice of delayed cord clamping, defined by ACOG as occurring >30 s after delivery [29], is becoming more common. Studies have shown benefits of delayed cord clamping for preterm infants; however, benefits in term infants appear to be marginal [30]. Delays in collection have been associated with smaller volumes and corresponding TNCCs and increase collection failures owing to clotting [16,31,32]. Because blood flow within the umbilical vessels immediately after birth is influenced by multiple physiologic factors [33], the true impact of delayed cord clamping at a specified time point on collection quality is difficult to assess. Early studies demonstrate that delayed clamping beyond 1 min is associated with loss of cord blood available for banking. It is apparent that further studies are needed to better understand the impact of cord clamping on the neonate, but it is also clear that this will be an ongoing discussion with important obstetric, perinatal, and banking implications. With the available data, it is recommended that delayed clamping is not practiced when cord blood is collected as a directed donation within a family for allogeneic transplantation in a sibling.

Processing and Cryopreservation

Many CB banks receive collections from distant sites, which results in potential delays in processing. Although stability of TNCC and CD34$^+$ content at room temperature have been reported to range from 24 to 96 h after collection [15,34–37], we and others have demonstrated small but significant losses of TNCC, CD34$^+$ cells, and CFU content at even earlier time points [24,38,39]. Based on these findings, our standard operating procedures at the CCBB involves triaging CBUs to allow for processing within 24 h of collection. Unrelated donor banking standards require cryopreservation to begin within 48 h of birth.

The general approach to processing CB is similar between banks, although some variations exist. Rubinstein et al. demonstrated that volume reduction achieved through removal of plasma and red blood cells (RBC) allows for more efficient processing and improved cell recovery after thaw [40]. Many banks continue to use the Rubinstein method, or variations of it, to achieve plasma and RBC depletion. Manual CB processing continues to be performed in some

banks, but an increasing number of banks are using automated systems for plasma and RBC reduction. Postprocessing, DMSO, typically in a final concentration of 10% along with 5% dextran or hydroxyethylstarch, is added as a cryoprotectant [41,42]. Cryopreservation occurs via controlled-rate freezing before storage in the liquid or vapor phase of liquid nitrogen for long-term storage at less than −180°C.

Cord Blood Unit Characterization

Banking standards require CB products to be tested and characterized extensively to assess the purity, potency, and sterility of the CBU. Testing in most banks includes assessing postprocessing TNCC, viability, viable $CD34^+$ cells, growth of CFUs, and sterility.

Assessing viability by any of several different methods is included in the banking standards for accreditation and is required for unit licensure. Postprocessed samples should contain at least 85% viable cells. Whereas fresh CB generally has high viability, insults to cells that can decrease viability include temperature changes, longer time to processing, and prolonged exposure to DMSO before cryopreservation [36,39,41], which may affect various cell populations within CB differently [36]. For example, decreases in viability may simply reflect cell death of mature granulocytes and may not reflect loss of HSCs.

$CD34^+$ is a surface marker of HSCs, and higher infused doses (cells per kilogram) have been associated with higher rates of engraftment, less transplant-related mortality, and improved overall survival (OS) [43]. As such, it is common practice to enumerate $CD34^+$ cells before cryopreservation and again after thawing for transplantation. Some transplant centers use the total $CD34^+$ cell dose in CBU selection. However, significant interlaboratory variability exists [44−47]. Efforts to standardize $CD34^+$ measurements led to the development of guidelines by the International Society for Hematotherapy and Graft Engineering [48]. This "dual-platform" method determines the percentage of $CD34^+$ cells by flow cytometry and measures the leukocyte count using an automated cell counter. Subsequently, "single-platform" approaches have been developed that enumerate $CD34^+$ cells using flow cytometry [49,50]. The viable $CD34^+$ content can be measured indirectly by using the percentage of viable cells to adjust the total $CD34^+$ dose. The presence of total $CD34^+$ cells, however, does not assess the viability and overall potency of a given CBU. This led to interest in measuring the viable $CD34^+$ content directly. FDA-cleared kits to enumerate viable $CD34^+$ cells have become more widely available and adopted for use by many CB banks. In fresh CB, total and viable $CD34^+$ content correlates closely [51−53], whereas there is more variability in thawed samples [51]. Use of the viable $CD34^+$ in CBU selection will require further standardization of the methods by the banking community.

The CFU assay, which requires viable cells to multiply and differentiate, is considered by many to be the best measure of CB potency. Studies demonstrated that CFU dose is strongly predictive of neutrophil and platelet engraftment and improved survival [54−58]. Identification and enumeration of colony types (CFU−granulocyte macrophage, CFU−granulocyte/erythrocyte/monocyte/megakaryocyte, and burst forming unit-erythroid) are performed by some banks, but specifications for these parameters are unknown. Despite the ability to assess potency, the CFU is a time-consuming assay that typically provides results 2 weeks later. Similar to measuring $CD34^+$ content, there are also issues with standardization among laboratories [59,60]. These issues have precluded its widespread use. Automated scoring systems and 7-day CFU assays have been developed to address these issues and are becoming more commonly used. There have also been focused efforts to develop alternate measures of potency that would provide results rapidly. Enumeration of CFUs using a thawed contiguous segment has been shown to be representative of the CB product and has been used to assess potency [61].

Aldehyde dehydrogenase (ALDH) is an intracellular enzyme found in high concentration in HSCs and can be measured by a flow cytometry−based assay. Cells scoring positive ($ALDH^{br}$) are viable and likely to correlate with HSC content of a graft [62]. $ALDH^{br}$ activity strongly correlated with CFUs and with speed of engraftment in autologous transplant recipients [63−66]. This suggests that $ALDH^{br}$ content of a CBU may predict potency. In fresh CB, $ALDH^{br}$ correlates well with TNCC, CFU, and $CD34^+$ content [67]. However, potency of a CB graft is best assessed on the thawed product, thereby reflecting any potential injury incurred as a result of cryopreservation and thaw. Therefore, we developed a potency assay for CBU release that can be performed at the time of confirmatory testing using a segment attached to a cryopreserved CBU. The assay enumerates $ALDH^{br}$, $CD34^+$, $CD45^+$, glycophorin A^+, viability (7-AAD^+), and CFUs from the thawed segment (Fig. 10.4). Our study demonstrated a strong correlation between $ALDH^{br}$ and CFUs measured on the segment ($r = 0.78$). However, the correlation between $CD34^+$ (as a percentage of viable CD45 cells) and CFUs was weaker ($r = 0.25$). Comparisons between cryopreserved segments and entire unit demonstrated strong overall correlation ($r = 0.88$). We also observed faster

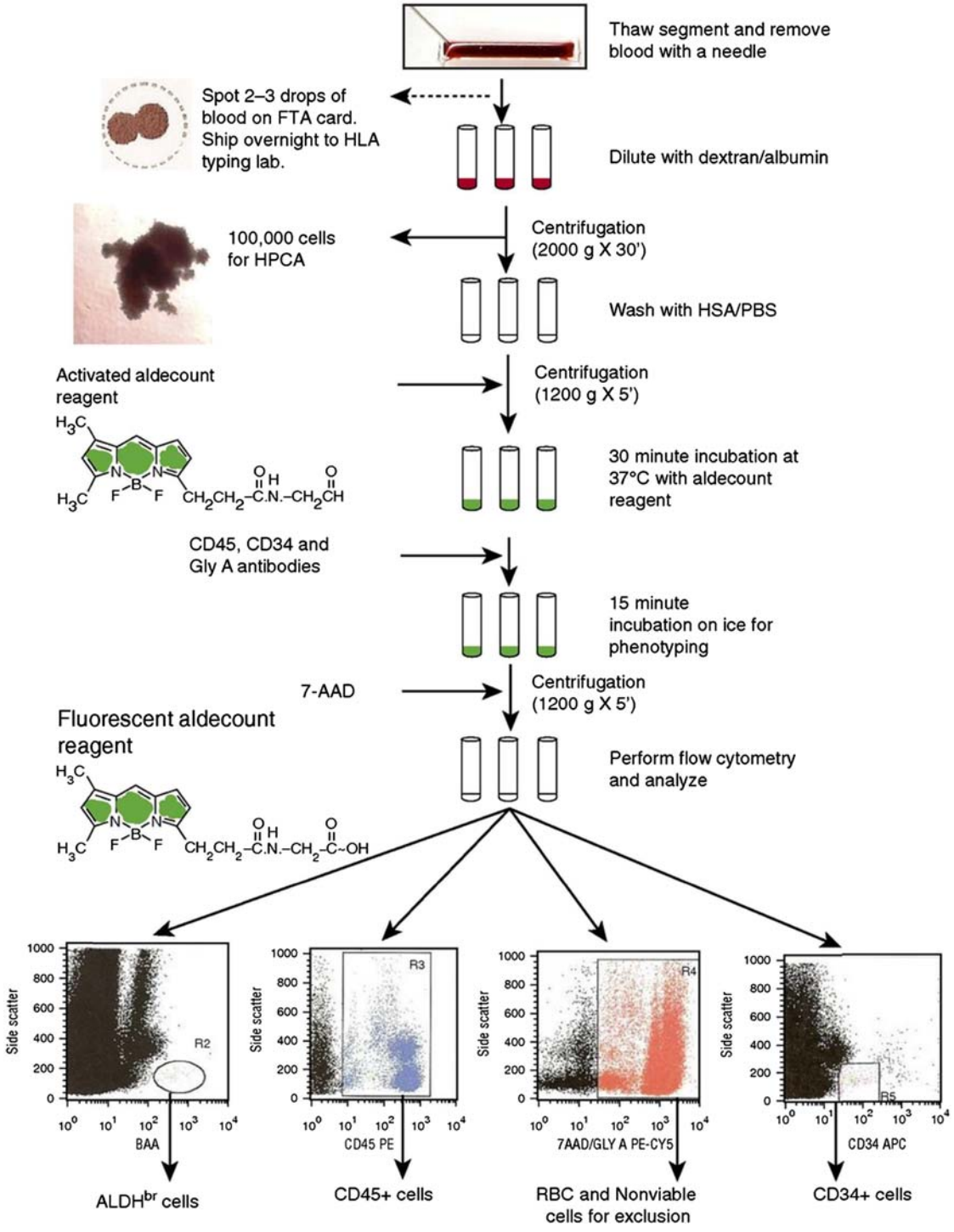

FIGURE 10.4 Flowchart of the ALDH potency assay performed on attached segments of CBUs requested for confirmatory HLA typing for donor selection. *7-AAD*, 7-aminoactinomycin D; *ALDH*, aldehyde dehydrogenase; *FTA*, fast technology analysis; *GlyA*, glycophorin A; *HLA*, human leukocyte antigen; *HPCA*, hematopoietic progenitor cell assay; *HSA/PBS*, human serum albumin/phosphate-buffered saline; *RBC*, red blood cells. *Used with permission from Shoulars KW, Noldner P, Troy JD, et al. Development and validation of a rapid, aldehyde dehydrogenase bright-based, cord blood potency assay. Blood 2016;127.*

engraftment in patients who received CB grafts with higher $ALDH^{br}$ measured on the segment ($P = .03$) [68]. Our findings demonstrated that the assay can serve as a surrogate for postthaw measurements to assess the potency of a potential CBU graft. Based on these findings, we have been using this assay before releasing CBUs from the CCBB to the transplant centers.

Finally, to prevent potential transmission of microbial agents to transplant recipients, all CB banks perform sterility assays on samples of processed CBU before cryopreservation. Screening units for bacterial and fungal contamination is most commonly performed using automated culture systems with high detection capabilities [69,70]. Rates of contamination reported in the literature have been variable, but generally range from 2% to 5% [15,71,72]. CBUs screening positive in sterility assays are excluded from public bank registries. However, directed donor units that are contaminated with bacteria may be stored and used for transplantation after the related recipient is covered with appropriate antibiotics before and after the infusion.

CLINICAL USES OF UMBILICAL CORD BLOOD

Cord Blood Transplantation for Hematological Malignancies

Most of the early clinical experience with CBT was in treating children with hematologic malignancies. The Cord Blood Transplantation Study trial, the first prospective multicenter phase II study of unrelated CBT, enrolled patients with pediatric leukemias, adult leukemias, pediatric immunodeficiencies, and infant leukemia, and pediatric patients with certain inherited metabolic diseases in a series of phase II clinical trials. The cohort of pediatric patients with hematologic malignancies were enrolled from 1998 to 2003 and received myeloablative conditioning (total body irradiation, cyclophosphamide, equine antithymocyte globulin) followed by infusion of an unrelated donor single CBU [12]. The results were encouraging and demonstrated that CB could provide adequate reconstitution of hematopoiesis and immune reconstitution with relatively low rates of GvHD. Relapse-free, 5-year survival was about 50% in this cohort of patients. Results of the adult stratum were less promising, however, likely because of the high number of comorbidities in end-stage patients and also because a typical CBU lacked sufficient cells to engraft reliably in an adult-sized individual. To overcome this obstacle, the team at the University of Minnesota pioneered the use of "double CBT" (i.e., two CBUs for one transplant) and reported improved rates of engraftment and survival in adults [11]. From 2005 to 2012, the Blood and Marrow Transplant Clinical Trials Network, in collaboration with the pediatric cooperative group, Children's Oncology Group, conducted a multicenter, randomized trial comparing one or two CB grafts in children with hematologic malignancies [14]. They found that in adequately dosed children, a single CBU was sufficient for transplant. Of note, chronic GvHD was increased in recipients of double cords compared with recipients of single CBUs. Patients experienced low rates of relapse, about 12%, in both treatment arms, and overall event-free survival was 70%. Milano et al. reported in an analysis of 582 patients that patients with detectable minimal residual disease before transplant had a significantly lower risk of relapse when CB grafts were used, compared with matched and mismatched unrelated adult donor grafts [73]. These results added to growing evidence that CB grafts may be able to provide a more potent antileukemic effect compared with other graft sources.

Cord Blood Transplantation for Nonmalignant Hematological Diseases

CBT has also been used successfully to treat other diseases amenable to HSCT. This heterogeneous group of diseases includes primary immunodeficiencies (PIDs), congenital and acquired bone marrow failure (BMF) syndromes, hemoglobinopathies, and inherited metabolic diseases (discussed separately subsequently). Generally, special considerations for these patients with nonmalignant conditions include decreasing the risk of GvHD and minimizing toxicities caused by the preparative regimen. Although this led to interest in developing reduced intensity conditioning regimens for these patients, rates of graft failure were much higher with this approach and the use of myeloablative conditioning continues to be the standard of care. Exceptions to this include certain diseases that are highly chemosensitive (i.e., Fanconi anemia and dyskeratosis congenita) [74,75] and certain PIDs that require little or no conditioning [76,77]. With several decades of experience in CBT, many lessons have been learned in the care of these primarily pediatric patients with nonmalignant conditions. Importantly, excellent survival (>90%) is noted when related CB grafts are used [75,76,78–82], and GvHD is lower than in related bone marrow recipients [79,81,83]. Therefore, related CBT, when available, is considered the standard of care for patients with nonmalignant diseases [83].

Experience using unrelated donor CB grafts for patients with nonmalignant diseases has generally been successful, depending primarily on the underlying disease. Patients with immunodeficiencies receiving CBT have good outcomes in smaller reports [84–91]. Morio et al. reported a 5-year OS of 69% for 88 patients with PIDs [92] with improved survival noted in patients with Wiskott–Aldrich syndrome (82%). In that series, the most common cause of death was infection. Pai et al. examined outcomes of 240 infants with severe combined immune deficiencies

transplanted using matched related or alternative donors at 25 centers from 2000 to 2009 [76]. They and others observed comparable outcomes for patients receiving unrelated donor transplantation regardless of donor source [76,77]. Younger infants (aged less than 3.5 months) experienced improved outcomes compared with older infants. It is likely, with the wider availability of newborn screening, that children will be transplanted at an earlier age, which will further improve these outcomes. Outcomes after unrelated CBT for inherited and acquired BMF syndromes have not been robust, because of higher rates of primary graft failure. MacMillan et al. described the outcomes of 130 patients with Fanconi anemia [93]. They observed that although outcomes using unrelated CB grafts historically were disappointing, similar neutrophil recovery was seen in patients receiving unrelated bone marrow and CB grafts, a finding attributed to optimizing donor selection and chemotherapy regimens. Unrelated CBT was used to treat other inherited BMF syndromes such as dyskeratosis congenita, congenital amegakaryocytic thrombocytopenia, Diamond-Blackfan anemia, and osteopetrosis [75,94–96], but it has been associated with higher rates of graft failure [75,94]. Similar issues with engraftment were reported in 71 patients undergoing unrelated CBT for acquired severe aplastic anemia [97]. In the report from the European Society for Blood and Marrow Transplantation, the cumulative incidence of neutrophil engraftment was 51% at 60 days and a 3-year OS of 38% [97]. Significantly improved OS was seen in patients who received higher TNC doses ($>3.9 \times 10^7$ cells/kg) from the CB graft. It is important to acknowledge, owing to the rarity of these diseases, that these studies included patients from an earlier transplant era, which probably negatively influenced the results. It is likely that contemporary outcomes would be more robust, because of improvements in supportive care and donor selection. Unrelated CBT remains a viable donor option when matched related donors are unavailable.

Thalassemia major and sickle cell disease (SCD) are the most common hemoglobinopathies worldwide. Allogeneic HSCT is the only curative treatment and best outcomes occur if HSCT is performed early in life, before significant organ dysfunction has occurred. Importantly, patients with hemoglobinopathies are at increased risk for graft rejection owing to several factors such as marrow hyperactivity to compensate for chronic anemia, alloimmunization resulting from multiple transfusions, and lack of prior chemotherapy exposure, which leaves the patient immunocompetent immediately before transplant. Similar to other nonmalignant diseases, the use of related CBT is associated with excellent outcomes [98]. Unrelated CBT for hemoglobinopathies has been more challenging, as highlighted in several studies [99,100]. Ruggeri et al. reported the outcomes of unrelated CBT in 51 children with either thalassemia (N = 35) or SCD (N = 16). Most (76%) received myeloablative conditioning followed by infusion of primarily HLA-mismatched grafts (two to three loci: 50%). High graft failure was seen in this cohort (27 of 51 patients), which was strongly associated with the TNCC cell dose [100]. Given the lower available cell dose in CBUs, it is unsurprising that graft failure has been an issue for these patients in the unrelated donor setting.

Cord Blood Expansion Technologies

Extensive investigations are under way to manipulate or expand CB HSCs ex vivo with the goal of more rapid immune and hematopoietic reconstitution. Expansion techniques that are in clinical trials include a Notch ligand–based platform [101], a nicotinamide-based expansion approach (NiCord) [102], fucosylation [103], coculture with mesenchymal cells [104], Stem-regenin (SR1) [105], and UM171 [106]. Most are being tested in patients with hematologic malignancies, but there is a current phase I trial using NiCord to treat pediatric patients with hemoglobinopathies, with promising early results (ClinicalTrials.gov: NCT01590628) [107]. Likewise, the Notch expanded product is undergoing testing in a multicenter, phase II randomized trial as a bridge to engraftment after a standard CBT after myeloablative conditioning therapy. If successful, these approaches may ultimately be applied to unrelated CBT for patients with hemoglobinopathies, thus broadening the utility of CBT in these diseases.

CORD BLOOD THERAPIES FOR INHERITED AND ACQUIRED BRAIN DISEASES

Cord Blood Transplantation for Inherited Metabolic Disorders

Inherited metabolic disorders (IMD) are a heterogeneous group of genetic diseases. In most of these diseases, a single gene mutation causes an enzyme defect, which leads to the accumulation of substrates that are toxic and/or interfere with normal cellular function. Many affected patients appear normal at birth. During infancy, however, they begin to exhibit disease manifestations, often including progressive neurological deterioration associated with absent or abnormal brain myelination. The ultimate result is death in later infancy or childhood.

In the 1960s, Elizabeth Neufeld demonstrated that coculture of fibroblasts from patients with two different IMDs (Hunter syndrome and Hurler syndrome) cross-corrected each other [108]; this established the basis for enzyme replacement therapy (ERT) and cellular therapy for that purpose. ERT is available for selected IMDs and can be effective in ameliorating certain systemic disease manifestations, although there are limitations. ERT is unable to cross the blood–brain barrier effectively and therefore does not alter the progression of neurologic symptoms [109,110]. Intrathecal ERT [111] and gene therapy [112] are being investigated to attempt to address this shortcoming. However, the only effective therapy to halt neurologic progression of disease is allogeneic HSCT, which serves as a source of permanent cellular ERT [113] providing missing enzyme throughout the body, including the peripheral tissues and the central nervous system [114,115]. Donor microglia cells, which are of myeloid origin, are thought to be the source of ERT after HSCT [116], in addition to acting as normal scavengers in the central nervous system [116–118]. It is also possible that donor cells exert antiinflammatory and proneurogenic effects through paracrine signaling. The timing of migration to, and engraftment of, donor-derived microglia cells in the brain after HSCT is not known. Based on clinical observations, however, it is likely several months after hematologic engraftment.

HSCT is indicated for a subset of IMDs including lysosomal storage diseases, peroxisomal storage diseases, and a few select others. The first HSCT for an IMD was performed in 1980 in a 1-year-old child with Hurler syndrome (mucopolysaccharidosis [MPS], type 1), a lysosomal storage disease, using bone marrow from his parents [119]. Since then, more than 500 transplants have been performed worldwide in patients with Hurler syndrome, which makes it the most transplanted and well-studied IMD. Numerous reports have demonstrated the efficacy of HSCT in Hurler syndrome, including improvements in neurocognitive function, joint integrity, motor development, growth, hydrocephalus, corneal clouding, cardiac function, hepatosplenomegaly, hearing, visual and auditory processing, and OS [120–126]. Despite improvements in both symptoms and life expectancy, survivors experience a variable degree of residual disease burden [127]. Factors associated with superior clinical outcomes include transplantation early in the disease course and the ability to attain full donor chimerism with normal enzyme levels [126,128]. Among patients receiving CBT for Hurler syndrome, a shorter interval between diagnosis and CBT (<4.6 months [82%] versus >4.6 months [57%]) and a conditioning regimen containing busulfan and cyclophosphamide (75% versus 44% using other regimens) are associated with a significantly higher event-free survival [129]. Studies also demonstrate that unrelated, noncarrier CB has advantages in the transplantation of MPS in terms of achieving both full-donor chimerism and normal enzyme levels compared with other donor sources [56,120,128,130–132]. Thus, CB has been identified as an attractive source for HSCT in Hurler syndrome and other MPS types for which HSCT is indicated.

CBT has also been used to treat patients successfully with certain leukodystrophies, a group of disorders caused by genetic defects in the production or maintenance of myelin, including adrenoleukodystrophy [133,134] metachromatic leukodystrophy [135,136], and Krabbe disease (globoid leukodystrophy). Prognosis is strongly affected by the stage of the disease at the time of transplantation; children who undergo HSCT in presymptomatic or early disease stages fare better than those in symptomatic or advanced stages [137]. As such, HSCT is generally reserved for patients with presymptomatic or early disease. This is particularly challenging in the case of early infantile Krabbe disease. Symptoms typically become evident during the first 6 months of life, although there is evidence that damage occurs even prenatally. Krabbe disease is caused by mutations in the lysosomal enzyme galactosylceramidase, which then leads to an accumulation of psychosine followed by apoptosis of myelin-forming cells in the central and peripheral nervous systems. Affected babies develop irritability, spasticity, developmental regression, and seizures. Progression of symptoms is rapid and unrelenting, leading to death typically within 2 years. In 2005, the outcomes of 25 babies with Krabbe disease who received CBT were reported. The cohort included 11 presymptomatic newborns (aged <1 month of life) along with 14 infants transplanted after the onset of symptoms [132]. Survival at 3 years was dramatically improved in the presymptomatic babies (100% versus 42.8%). The presymptomatic babies also exhibited substantial neurodevelopmental gains whereas symptomatic infants stabilized without improvement. Nonetheless, some degree of gross motor function deficit became apparent in all children. An analysis of late outcomes showed that babies transplanted younger than age 30 days had superior outcomes compared with those transplanted at age greater than 30 days [138]. Results from this and other studies [87,132,136] reinforce the importance of early diagnosis and treatment. Given the time-sensitive nature, newborn screening programs for Krabbe disease have been instituted in a portion of US states with further programs in development.

One issue inherent in HSCT is that disease progression can continue in the initial few months after transplantation. While waiting for sufficient numbers of donor cells to engraft in the brain and produce adequate enzyme levels, patients can experience further loss of neurologic function, and most patients are left with some residual and irreversible neurologic impairment. Even when complete donor hematopoietic chimerism and normal serum enzyme levels are obtained and survival is extended for decades, emerging long-term data suggest that eventual neurologic

decline, particularly in motor function, commonly occurs later in life. Additional approaches are necessary to address the multifaceted tissue pathology fully in these diseases and normalize functional outcomes for patients. Augmented cellular therapies, such as CB-derived microglial-like cells (DUOC-01) [139–141] and others [142], gene therapies [112], supplemental enzyme therapy [143], and chaperone therapy, alone or combined with HSCT, are all being investigated in the laboratory and/or clinic for that purpose.

INVESTIGATIONS IN THE TREATMENT OF ACQUIRED BRAIN INJURIES WITH UMBILICAL CORD BLOOD

Aside from acting as cellular enzyme replacement, donor CB cells in patients with IMDs may also have a role in replacing damaged cells, secreting supportive factors, and immunoregulation. These additional possible mechanisms led to the hypothesis that CB might also be beneficial in patients with acquired brain injuries. CB cells have been investigated in preclinical models of stroke, neonatal hypoxic-ischemic encephalopathy (HIE), cerebral palsy, traumatic brain injury, and spinal cord injury. Numerous animal models have demonstrated neuroprotection [144], neovascularization [145], and neuronal regeneration [145] after xenogeneic CB administration, leading to both neurological and survival benefits [144,146–151]. Based on these observations, early-phase clinical trials of CB are under way in human patients with acquired brain injuries, using either autologous or allogeneic CB cells.

Hypoxic Ischemic Encephalopathy

Neonatal HIE results from an acute lack of oxygen to the infant brain, typically attributed to events during labor and delivery. Therapeutic hypothermia is considered the standard of care for babies who meet criteria for moderate to severe HIE. Despite this intervention, moderate to severe HIE is fatal in approximately 25% of patients and causes neurologic sequelae in an additional 25%. In a phase I trial conducted at Duke that enrolled neonates with HIE, fresh autologous CB (volume- and RBC-reduced) was infused within the first 48–72 h of life in one, two, or four doses of $1–5 \times 10^7$ nucleated cells/kg in babies with moderate to severe encephalopathy who qualified for systemic hypothermia [152]. Those babies ($n = 23$) were compared with a concomitant group of babies at Duke who received only therapeutic hypothermia ($n = 83$). Infusions were found to be safe in those critically ill babies. The babies who received cells had increased survival rates to discharge (100% versus 87%; $P = .12$) and improved function at 1 year of age (72% versus 41% with development in the normal range; $P = .05$). Based on these findings, a phase II, randomized, placebo-controlled multicenter study is under way to investigate further the utility of CB infusion in conjunction with therapeutic hypothermia for babies with HIE.

Cerebral Palsy

In most cases, cerebral palsy results from an in utero or perinatal brain injury such as stroke, hypoxic insult, or hemorrhage. Clinical outcomes of patients with cerebral palsy are widely varied, ranging from mild limitations in advanced gross motor skills to severely limited self-mobility despite the use of assistive technology. The use of CB in children with cerebral palsy is being investigated in clinical studies. Romanov et al. reported results of 80 pediatric patients who received multiple intravenous doses of allogeneic, ABO-matched, HLA-unmatched CB. Improvements were noted in children who received at least four doses; however, there was no control group for comparison [153]. Many children with cerebral palsy are expected to make some gains over time, which is an important factor to consider in study design. A double-blind study was conducted in Korea in 93 children who were randomized to receive erythropoietin, erythropoietin plus cyclosporine plus allogeneic CB ($\geq$4/6 HLA matched, $\geq 3 \times 10^7$ TNC/kg), or placebo [154]. They reported greater improvements in cognitive and select motor functions in children who received CB and erythropoietin versus controls; higher cell doses were associated with greater improvement. A CB-only group was not included.

In the United States, investigations of CB in children with cerebral palsy have focused on intravenous infusions of autologous CB that had been banked at the time of the child's birth. An initial safety study of 184 infants and children with cerebral palsy (76%), congenital hydrocephalus (12%), and other brain injuries (12%) identified a temporary hypersensitivity reaction (i.e., hives and/or wheezing) in approximately 1.5% of patients as the only side effect [14]. A randomized, double-blind, placebo-controlled study was subsequently conducted in 63 children aged 1–6 years to evaluate the efficacy of this approach. In that study, there was no difference in motor improvement

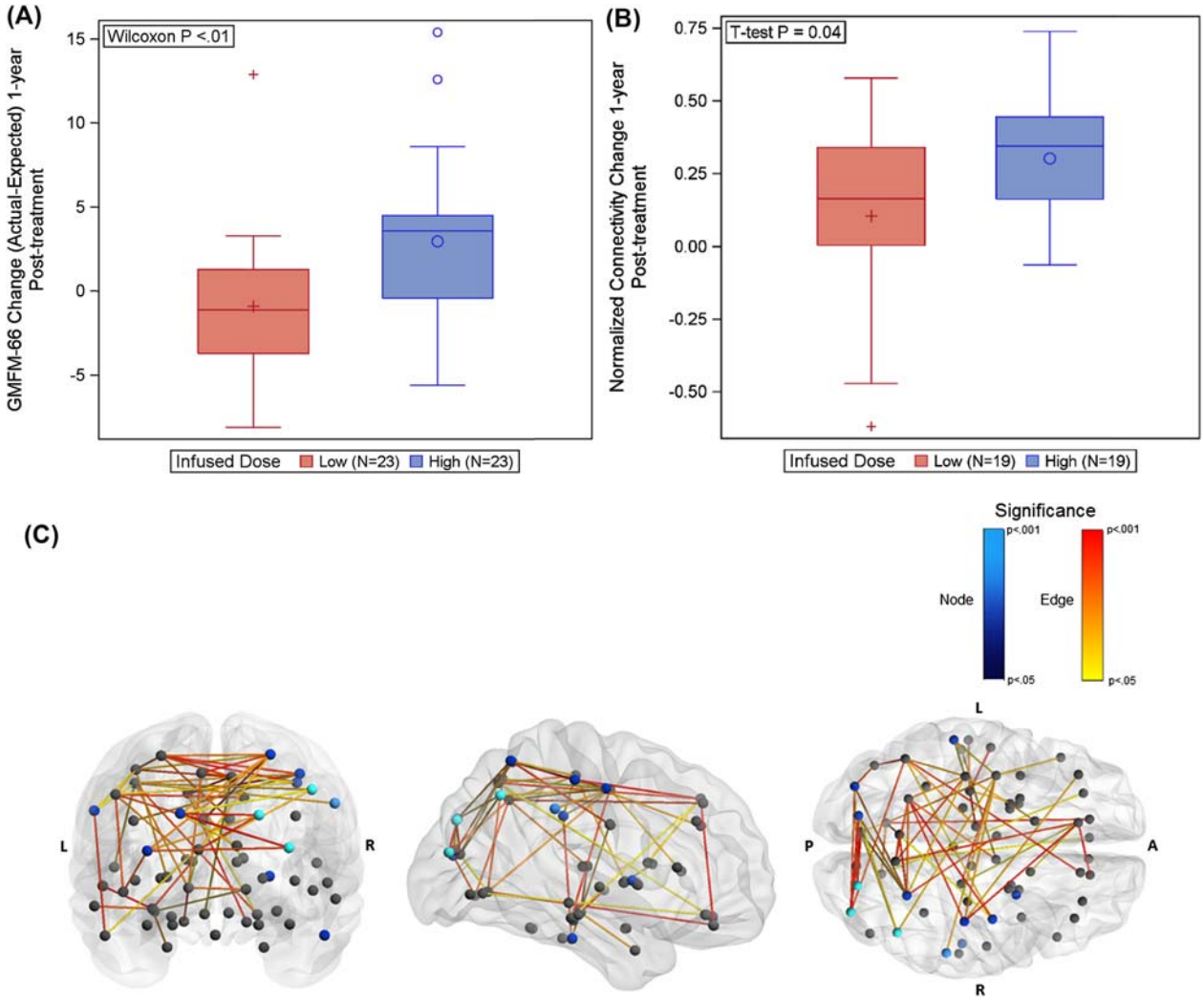

FIGURE 10.5 **Improvements in motor function after appropriately dosed autologous cord blood infusion: correlation with increased whole-brain connectivity.** Gross motor function and brain connectivity 1 year after autologous cord blood treatment by cell dose. (A) Observed−expected GMFM-66 scores 1 year after treatment in patients aged greater than 2 years at the time of autologous cord blood infusion. (B) Change in normalized whole-brain connectivity 1 year after treatment. (C) Connectome representation. Nodes and edges included are those demonstrating significantly increased improvement in children receiving high doses compared with those receiving low doses, as indicated by the *color chart*; insignificant nodes are shown in *gray*. Representative nodes in the sensorimotor network with significant changes correlated with improvement in GMFM-66 scores include the pre- and postcentral gyri, basal ganglia, and brain stem. *GMFM-66*, Gross Motor Function Measure−66.

between study groups as a whole. However, patients receiving a previously cryopreserved nucleated cell dose of $\geq 2.5 \times 10^7$ cells/kg demonstrated greater improvement of motor function and normalized whole brain connectivity than did subjects receiving smaller cell doses, which is consistent with the minimum dose used for allogeneic HSCT (Fig. 10.5) [155].

Stroke

Stroke represents a significant public health concern and is a leading cause of morbidity and mortality among adults. Studies of cell therapy in older patients who have experienced a stroke have focused on autologous bone marrow−derived cells. The only published randomized trial, conducted in 120 patients in India, administered autologous bone marrow mononuclear cells (mean, 280×10^6 cells) as a single intravenous dose 7−30 days after an acute stroke [156]. There was no difference in functional outcomes or infarct volume between groups at 6 months. Additional studies are under way or planned using autologous and allogeneic cells [157]. There are concerns regarding

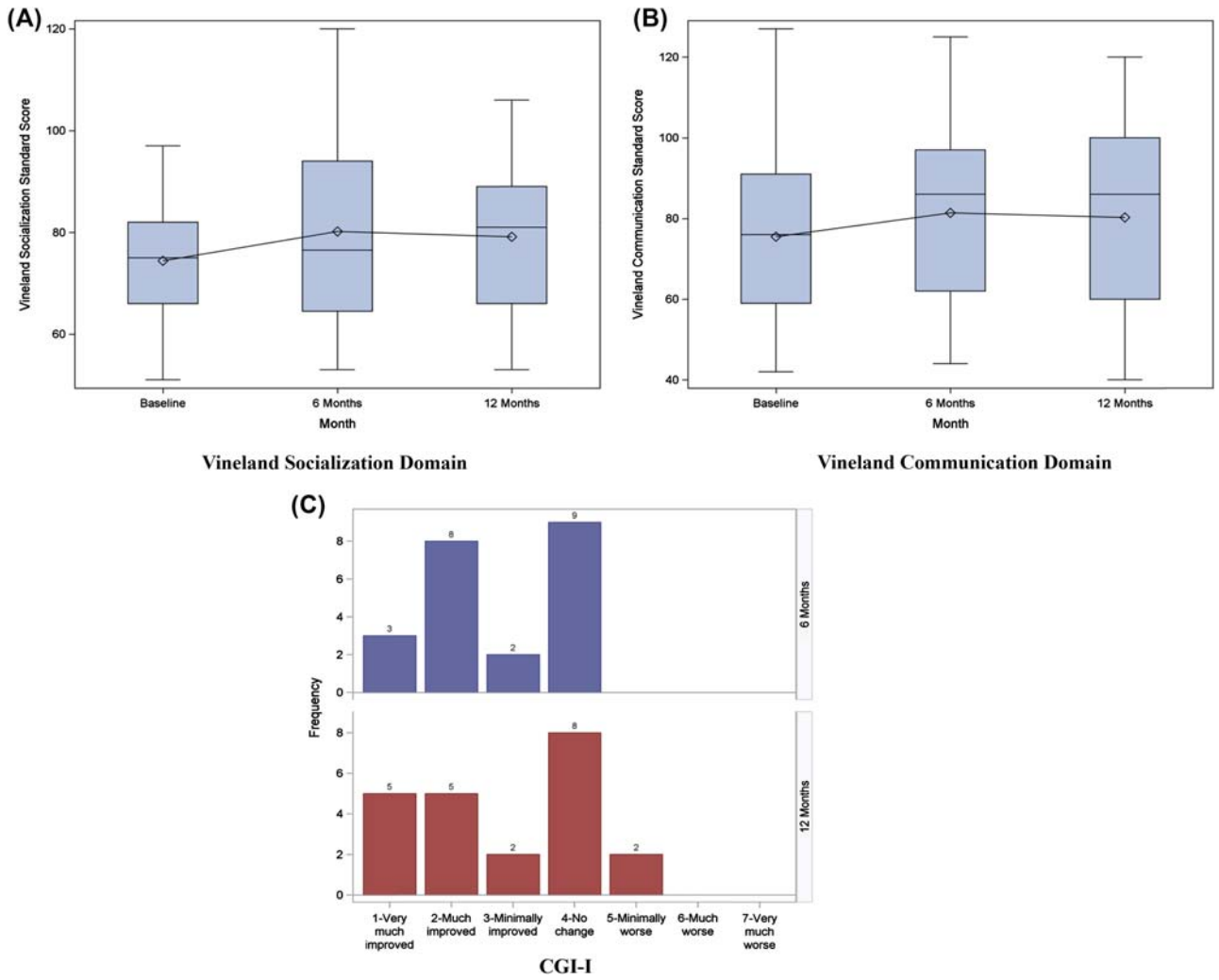

FIGURE 10.6 **Changes in ASD symptoms after autologous CB infusion.** (A) Vineland-II socialization domain standard scores at baseline and 6 months after infusion ($P = .02$ baseline to 6 months). (B) Vineland-II communication domain standard scores at baseline and 6 months after infusion ($P < .01$ baseline to 6 months). (C) Distribution of CGI-I scores at 6 (*blue*) and 12 (*red*) months after infusion. Sample sizes are N = 25 for baseline and 6-month time points and N = 22 at 12 months. *ASD*, autism spectrum disorder; *CB*, cord blood; *CGI-I*, clinician assessment. *Used with permission from Dawson G, Sun JM, Davlantis KS, et al. Autologous cord blood infusions are safe and feasible in young children with autism spectrum disorder: results of a single-center phase I open-label trial. Stem Cells Transl Med 2017;6(5):1332–9.*

the functionality of autologous bone marrow–derived cells, because they must be collected from a typically ill, elderly patient, and the feasibility of early administration may not be possible if the cells must be cultured or processed for any length of time. Therefore, a CB-derived, off-the-shelf therapy is an attractive alternative to autologous bone marrow. A phase I safety study administering ABO-matched, HLA-unmatched allogeneic CB intravenously to adults within 3–10 days of acute ischemic stroke completed accrual and demonstrated no acute safety issues related to the CB infusion [131]. A phase II randomized, placebo-controlled, blinded study to evaluate efficacy has enrolled patients at four centers.

Autism Spectrum Disorder

Intravenous infusion of CB is also being investigated in young children with autism spectrum disorder (ASD), a neurodevelopmental disorder with onset in early childhood. ASD is characterized by impairments in social communication, a restricted range of activities, and repetitive behaviors [158]. Evidence suggests that ASD likely results from a complex interplay between genetic and environmental risk factors, potentially mediated through inflammatory and/or immune processes. One hypothesis regarding the development of ASD is that immune-mediated

changes in fetal brain cytokine profiles may result in abnormal development in the central nervous system, either directly or indirectly via microglial activation. Abnormalities in the number, function, and gene regulation of microglia as well as in localized brain inflammation, pathological astrocyte activation, and synaptic dysfunction have all been described in various models of ASD [159–161]. Cellular therapies are being investigated for their potential to reduce ongoing inflammation via cell-mediated immunomodulation, provide neuroprotection via molecular mechanisms, and/or restore functional synaptic pathways. These potential mechanisms are thought to occur via paracrine effects.

Clinical trials of cell therapy in patients with ASD are still in the early phases; a handful of exploratory studies using various cell sources are under way in several different countries. The open-label phase 1 DukeABCs trial examined the safety and tolerability study of a single intravenous infusion of autologous CB in 25 children aged 2–5 years who had ASD (ClinicalTrials.gov: NCT02176317) [162]. Participants received a single CB infusion (median infused dose, 2.6×10^7/kg; range, $1.0–8.1 \times 10^7$/kg) with no immunosuppression. The infusions were safe, with no serious adverse events and occasional allergic reactions and irritability reported. Improvements in ASD symptoms were observed on caregiver-completed measures (Vineland Adaptive Behavior Scales–Second Edition and Pervasive Developmental Disorder Behavior Inventory), clinician assessment (Clinical Global Impressions scale [CGI-I]), and computerized eye-tracking assessments (Fig. 10.6). Positive changes, including increased social communication skills and receptive/expressive language and decreased repetitive behavior and sensory sensitivities, were observed at 6 months and maintained at 12 months after infusion. A phase 2 double-blind, randomized, controlled study is under way to evaluate the efficacy of autologous or allogeneic CB therapy versus placebo in children with ASD (ClinicalTrials.gov: NCT02847182).

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CHAPTER

11

Induced Pluripotent Stem Cells

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INTRODUCTION

The capacity for unlimited self-renewal and the potential to differentiate into any cell in the body made embryonic stem cells (ESCs) a valuable research tool. However, before the mid-2000s, mouse ESCs, which were first derived in 1981, were more commonly used in research laboratories owing in part to the relative ease with which they could be maintained and expanded compared with the techniques used at the time to culture humans ESCs (hESCs). In 2003, the National Institutes of Health jump-started the field by sponsoring seven hESC laboratory courses across the United States, which trained a cohort that went on to use the technology in their own laboratories. Technological advances in the culture of hESCs such as single-cell passaging [1] and feeder-free media and substrata allowed for more laboratories to culture hESCs successfully. However, there has been no advance more important than the process of reprogramming adult somatic cells into induced pluripotent stem cells (iPSCs). That discovery fundamentally changed the stem cell field.

The production of iPSCs was first reported by Kazutoshi Takahashi and Shinya Yamanaka using adult mouse fibroblasts in 2006 and was immediately hailed as a ground-breaking discovery [2]. The introduction of four transcription factors, *Oct3/4* (*Pou5f1*), *Sox2*, *Klf4*, and *c-Myc* (*Myc*), was sufficient to transform adult fibroblasts into pluripotent stem cells. In 2007, Yamanaka repeated the process using human fibroblasts [3]. The process of reprogramming adult cells into cells functionally equivalent to hESCs has provided an unparalleled tool to study human development and generate cells for regenerative medicine applications. Yamanaka's discovery altered the dogma underlying our knowledge of cell biology and created new fields of study. In 2012, Shinya Yamanaka was awarded the Nobel Prize in Physiology or Medicine, along with John Gurdon, for the discovery that mature cells could be reprogrammed into cells capable of developing into all cells of the body. Gurdon is credited as the first to demonstrate that a cell nucleus could be reprogrammed to a pluripotent state through the process of somatic cell nuclear transfer [4,5].

MECHANISMS OF REPROGRAMMING

Since the first iPSCs study was published, scientists have gained a much better understanding of what was once mostly a black box process, in which the Yamanaka transcription factors were expressed as transgenes in cells and sometime later pluripotent colonies would emerge. Since the advent of iPSC technology, the molecular events underlying reprogramming have intrigued researchers. We now know that the reprogramming process is a positive feedback loop by which core transcription factors regulate the expression of a network of pluripotency-associated genes [6,7]. Early models postulated that reprogramming is a stochastic process in which most transgenic cells can initiate reprogramming but only a few achieve pluripotency [8]. Success in reprogramming may happen only stochastically when the required amount and balance of transgenic expression are achieved in a small subset of cells [9]. Subsequent studies using single-cell approaches at defined time points demonstrated that after the initiation of transgene expression, there is a hierarchical phase with *SOX2* as the upstream factor in the gene expression

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hierarchy, meaning that endogenous *SOX2* expression was required before endogenous expression of other key pluripotency-associated genes [10]. Reprogramming itself has been used as a tool to explore the role of various factors systematically in the maintenance of pluripotency. For example, redundancies in the pluripotency network have been identified, such as replacing *SOX2* in the reprogramming process by the closely related *SOX1* and *SOX3* [11]. Krüppel-like factor 2 (*KLF2*) or *KLF5* can be used as substitutes for *KLF4* [12].

EPIGENETIC REMODELING

For reprogramming to occur, major epigenetic barriers must be overcome. Epigenetics refers to modifications of the genome that can affect the ability for genes to be expressed. The addition of a methyl (CH_3) group to the five prime carbon of the cytosine ring is a well-characterized example that typically results in transcriptional inhibition. Throughout the process of differentiation, the methylation pattern in the genome changes and stabilizes different cell states. As such, the methylation state of an adult somatic cell must be remodeled during the reprogramming process to unlock access to the pluripotency-associated gene network. Many of these genes are hypermethylated in donor cell types but are hypomethylated in iPSCs [13]. The reprogramming factors do not affect DNA demethylation directly, so modifications of DNA methylation are likely a secondary effect of transcription factor induction. Achieving pluripotency also requires considerable histone remodeling [14].

Some studies have raised concerns that reprogrammed cells retain an "epigenetic memory" of the somatic tissue from which they were originally derived [15,16]. However, studies have indicated that epigenetic differences account for only a small fraction of the variability among iPSCs and ESCs [13,17]. In addition, like the lead character in the movie *Memento* (https://en.wikipedia.org/wiki/Memento_(film)), the epigenetic memories of iPSCs are short-term and easily lost; it has been shown that time in culture reduces the epigenetic differences among iPSCs [16,18]. This suggests that the global epigenetic patterns of iPSCs stabilize over time, although there are enduring hot spots of variation, such as at imprinted regions [13,19]. Detailed analysis of global gene expression and DNA methylation patterns reveals that variability among ESCs and iPSCs occurs largely because of variations among individual cell lines rather than differences among classes of pluripotent stem cells [13,17,20–22].

REPROGRAMMING TECHNIQUES

The reprogramming process initially was developed using retroviral transduction. Starting from a candidate pool of 24 factors considered to have important roles in pluripotency, Yamanaka reported that the combination of *Pou5f1*, *Klf4*, *Sox2*, and *Myc* was sufficient to produce iPSC colonies from adult mouse fibroblasts. Because the first iPSC lines were created with retroviral vectors that integrated within the host genome, there was concern that uncontrolled integration could disrupt tumor suppressor genes or activate oncogenes through the process of insertional mutagenesis. Of additional concern was that one of the Yamanaka factors, Myc, is an oncogene itself and that reactivation of the transgenes could result in tumor formation. Indeed, 20% of the offspring of chimeric mice derived from iPSCs that were created using retrovirus-developed tumors [23].

Because of the potential tumorigenicity of cells reprogrammed with integrating vectors, there is general consensus that the use of reprogramming methods that integrate transcription factors into the host genome is ill-advised if the cells are planned for clinical use. However, tools available to reprogram cells have rapidly evolved, and now a variety of nonintegrating alternatives exist and are widely practiced [24]. Integration-free methods rely on the fact that transcription factors used to reprogram the cells are necessary only during the early stages of reprogramming, after which awakening of the endogenous pluripotent machinery is sufficient to sustain the pluripotent state. The Sendai virus is a commonly used, nonintegrating method of reprogramming (see Fig. 11.1) [25]. The RNA virus does not translocate to the nucleus and is diluted with each cell division. In addition, episomal vectors can be used to deliver the Yamanaka factors; however, it has been demonstrated that episomal vectors can sometimes integrate into the host genome, so iPSC clones must be subjected to genomic analysis [24]. Another popular method for reprogramming is the introduction of synthetic messenger RNAs (mRNAs) [26]. The challenge with mRNA reprogramming is the need to repeat applications [27].

The efficiency of the first human reprogramming experiments was less than 0.01%, but reprogramming efficiencies of greater than 1% are common. Generally, the methods only need to be efficient enough to produce a few clones of iPSCs; because reprogramming is usually reproducible and much of the effort involved in

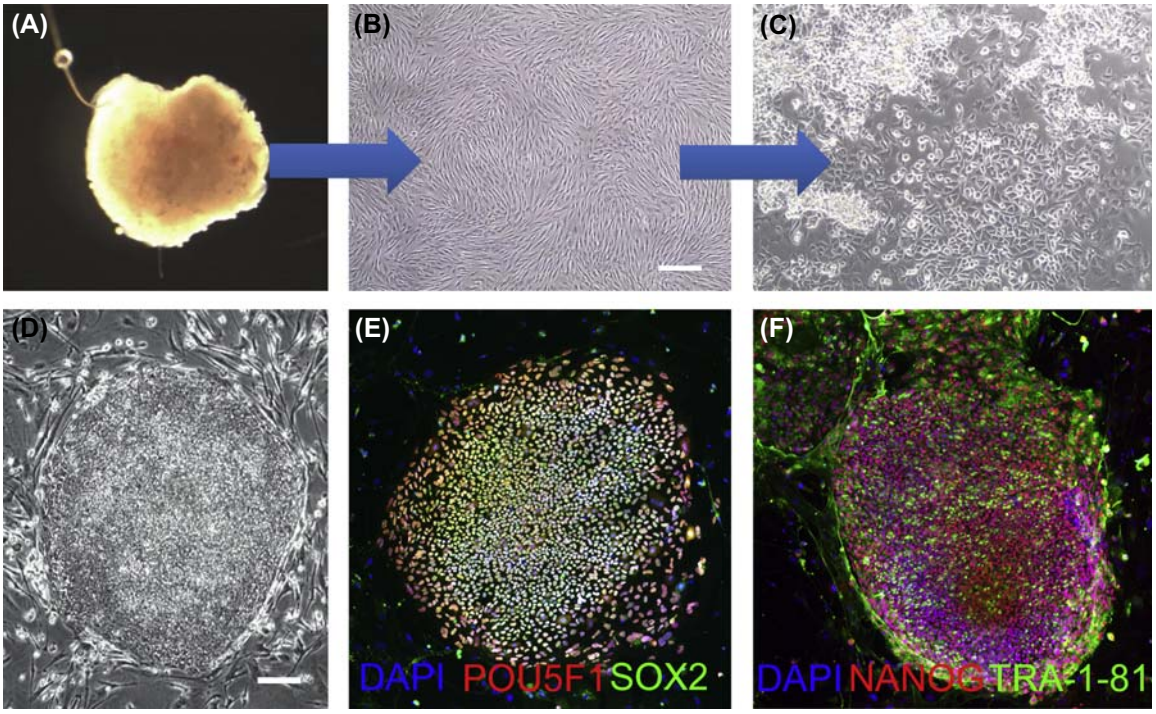

FIGURE 11.1 **Reprogramming human dermal fibroblasts using Sendai virus to deliver the four transcription factors**. A biopsy of dermal tissue (A) is obtained from the patient. The biopsy is then processed and dermal fibroblasts are isolated and cultured (B). After introduction of the Yamanaka factors, the morphology of the fibroblasts changes (C) as the cells undergo the reprogramming process. Finally, after a period of several weeks, colonies emerge (D) that express pluripotency markers, as shown by immunocytochemistry (E, F). Scale bar = 50 μm in (B, C) and 100 μm in (D–F).

reprogramming is in confirming pluripotency and characterization of the iPSCs, analysis of more than a small number of clones is not cost-effective. iPSCs are typically clonally derived, which means that single colonies of reprogrammed cells are picked and subcultured separately from other colonies. Clonal derivation preserves the genetic homogeneity of the iPSC line, a factor that is important in studying genetic disorders that are not present uniformly in the organism's cells. It has been argued that for high-throughput reprogramming, it is necessary to skip the clone-picking step and combine multiple clones [28]. This approach may be appropriate for normal cell lines, although starting populations such as fibroblasts and blood cells are mosaic [29]. Mixture of clones will lead to fluctuations in the subsequent cultures as they proliferate, because some iPSC clones divide slightly faster or are more resistant to stress than others. Many genetic diseases are also mosaic, so a single blood sample or a culture of fibroblasts will yield both normal and mutant iPSC clones. If clones are combined, they would need to be later subcloned and characterized.

INDUCED TRANSDIFFERENTIATION

The same principle of transcription factor–based reprogramming has been applied to direct transformation of one cell type to another. The process of transdifferentiation, also known as lineage reprogramming or direct lineage conversion, can bypass the pluripotent state when converting one cell type to another. Fibroblasts can be transdifferentiated into terminally differentiated cell types such as neurons [30] or cardiomyocytes [31] without using iPSCs. This approach is potentially useful for obtaining mature cell types for which there is currently no robust differentiation scheme. In addition, transdifferentiation is relatively fast compared with iPSC differentiation, which makes it more feasible to screen many individuals in a short period. Bioinformatic analysis can be used to predict what transcription factors are needed to convert one cell type into another [32], although sometimes the same transcription factor is active in more than one cell type, which leads to cells that are hybrids of two or more cell types [33,34]. If the products of transdifferentiation are postmitotic, they might be less likely than iPSC derivatives to contain residual undifferentiated cells that could produce teratomas when used for cell therapy. Several clinical trials are under way using hESC- and iPSC-derived cell preparations, with no report of tumor formation.

There are also drawbacks that should be considered in using direct transdifferentiation of cells such as fibroblasts to mature cell types. Like reprogramming to iPSCs, this process is inefficient, and because mature cell types have a limited capacity to divide (neurons and cardiomyocytes are postmitotic), direct reprogramming requires a large input population and must be repeated each time more cells are required. Transdifferentiated mature populations are not clonally derived, which means that genetic manipulation and characterization of the resulting cell population are difficult or not feasible. These drawbacks may be mitigated by targeting an intermediate progenitor or stem cell that retains a capacity for proliferation but is limited in differentiation capacity; this allows for purification of the cell type desired, and elimination of any abnormally programmed cells [35].

GENOMIC STABILITY

A study examined the potential for genomic damage during reprogramming using whole-genome sequencing and concluded that the reprogramming process itself is not likely to introduce mutations with adverse side effects [36]. Another exome sequencing analysis of fibroblast and iPSC clones concurred and reported that almost all of the genomic variation in iPSCs originates from the cell population used for reprogramming [37]. However, after reprogramming, populations of iPSCs can drift with time in culture like any other cell type, because genetically aberrant subpopulations tend to divide more quickly or are more resistant to apoptosis or other forms of cell death. The types of genetic variations observed range from large-scale karyotypic abnormalities to copy number variations and point mutations. Common karyotypic changes in iPSCs and hESCs include trisomies of chromosomes 12, 17, and X, and duplications of subchromosomal regions on these chromosomes [38–40]. In addition, studies of trinucleotide repeat diseases including Friedreich ataxia and Fragile X syndrome have reported changes in the repeat length after reprogramming [41,42].

Because iPSCs are self-renewing, they never senesce and will continue to divide as long as they are in culture. Because mutations arise when cells replicate, it is inevitable that genomic abnormalities will accumulate if the iPSCs are cultured for long periods of time. Of particular interest are the appearance of deletions of the tumor suppressor TP53 in hESCs after prolonged time in culture [43] and indications that similar deletions occur in iPSCs after long-term culture [44].

APPLICATIONS OF INDUCED PLURIPOTENT STEM CELLS

There has been concern in the field that iPSCs are somehow inferior to hESCs for practical applications. However, a number of reports have demonstrated that hESCs and iPSCs are essentially indistinguishable [13,21,22]. A study on transcriptional and epigenetic comparisons using genetically matched hESC and iPSC lines revealed that hESCs and iPSCs are molecularly and functionally equivalent and cannot be distinguished by their gene expression profiles [45]. This means that all of the applications established for hESCs should be easily transferred to iPSCs (Fig. 11.2).

DISEASE MODELING

Perhaps the most attractive feature of iPSCs is that it allows scientists to study human diseases using human cells. Much progress in modeling human disorders has been made possible by pioneering work to develop robust protocols to generate specific cell types from human iPSCs, including neurons [46–48] and cardiomyocytes [49]. iPSCs have been used routinely to model genetic diseases including monogenic disorders and chromosomal and more complex genetic disorders. Animal model systems have been used to study those disorders for decades, but positive results in animals have not always translated to human studies. This should not be surprising because rodents diverged evolutionarily from humans almost 60 million years ago. For example, in the nervous system, understanding the mechanisms underlying neurological disorders and development of pharmaceutical interventions has lagged far behind disorders affecting other organs. Many highly anticipated drugs, such as those that target Fragile X syndrome [50] and amyotrophic lateral sclerosis (ALS) [51–53], have failed to demonstrate efficacy in human trials after promising preclinical work on animal models. In addition, neurological and psychiatric diseases are complex because they are rarely based on a single gene variant. Notably, many psychiatric diseases arise from mutations in noncoding regions [54,55]. In many cases, these noncoding regions either are not conserved in rodents or they

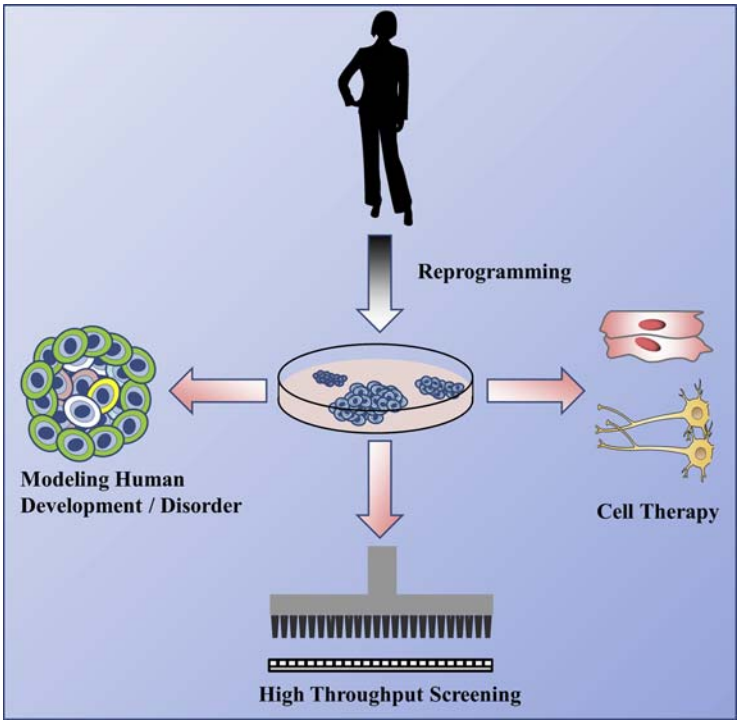

FIGURE 11.2 Applications of induced pluripotent stem cells (iPSCs). Human iPSCs can be used to study and treat human diseases. Aggregates of iPSCs can form organoids that recapitulate aspects of early embryonic development that can be used to model human developmental disorders. Genetically diverse iPSCs and their derivatives can be used in high-throughput screening to improve the preclinical development of pharmaceuticals. In addition, iPSCs can be used to derive therapeutically relevant cell types for autologous transplantation as a cell therapy to treat human diseases.

function differently. In contrast, iPSCs derived from patients with genetic disorders contain all of the complex genetic interactions that underlie the disease.

Timothy syndrome (TS) is a neurodevelopmental disorder that has been successfully investigated using patient-derived iPSCs [56]. TS is a rare monogenic form of autism caused by prolonged activation of the L-type $Ca_v1.2$ calcium channel. The prolonged channel activation results from a gain of function mutation leading to dysregulation of Ca^{2+} signaling in many cell types. One of the striking phenotypes of TS iPSCs is abnormal cortical development; they produce fewer upper-layer neurons and a higher proportion of lower-layer neurons. Interestingly, elevated Ca^{2+} signaling leads to upregulation of tyrosine hydroxylase in cortical neurons. Higher levels of tyrosine hydroxylase, an enzyme that is involved in the biosynthesis of catecholamines, ultimately lead to increased norepinephrine and dopamine production and are associated with aggression in TS patients. Using iPSCs as a model, it was discovered that roscovitine, an L-type channel blocker, can restore tyrosine hydroxylase expression and catecholamine production in TS iPSC-derived neurons. Notably, the transcription regulatory element of human tyrosine hydroxylase locus is not conserved in mice, and the catecholamine phenotype is not observed in a transgenic TS mouse model [57]. In this way, the TS iPSC model provided preclinical validations for future therapeutic development that would otherwise have been missed using only rodent models. Similarly, studies using iPSCs to model other monogenic forms of autism such as Phelan–McDermid syndrome [58], Rett syndrome [59], and Williams syndrome [60] identified human-specific phenotypes and discovered disease-causing pathways.

iPSCs are also useful for studying diseases for which there is evidence of inheritance but no specific mutations identified. For complex diseases such as idiopathic autism and schizophrenia, most cases lack a clear genetic basis. Deriving iPSCs from the patients preserves their genomes, and when differentiated into the relevant cell types, they can be used to study cellular phenotypes and molecular mechanisms without knowing the genetic cause. However, patients who have complex diseases usually present with a wide range of symptoms. Because obtaining information about a disease from patient iPSCs requires multiple patients with the same syndrome, careful consideration should be taken in selecting patients and study controls. For example, to study idiopathic autism, a research group selectively focused on patients who presented with the same clinical phenotype: early brain overgrowth. They observed an increased proliferation rate of the disease-associated iPSC-derived neural progenitor cells and determined that it was likely caused by dysregulation of the β-catenin/BRN2 cascade [61]. Selection of appropriate controls is extremely important to minimize non–disease relevant differences; because humans are genomically diverse, variations in individuals' iPSCs could contribute to differences in cellular behaviors that are not necessarily

disease relevant. For this reason, iPSCs from unaffected family members are usually used to study idiopathic disease to minimize variability owing to genomic diversity [62].

iPSCs are also valuable for validating human-specific disease-causing variants. Advances in sequencing technology have enabled an increase in genome-wide association studies (GWAS) to identify novel disease-causing candidate mutations. For many complex diseases, candidate mutations are found in noncoding regions and most are not evolutionarily conserved in animal models. iPSCs are an ideal platform for validating such candidate mutations identified through GWAS studies. An example is a single nucleotide polymorphism risk variant that appeared to contribute to the pathogenesis of Parkinson disease (PD) [63]. Using genome editing with Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 (Cas9), risk-associated alleles were introduced into wild-type iPSCs; by comparing the otherwise identical cell lines, researchers showed that the risk allele altered cis-regulation of the α-synuclein (SNCA) locus and led to an upregulation of SNCA expression. This variant mimicked a duplication of the coding region for SNCA that was known to be a familial cause of PD [64].

Methods have improved for culturing iPSCs in miniature "organoids" that can resemble the structure of human organs. When iPSCs are aggregated during their differentiation in vitro, they can create their own microenvironment and self-organize into three-dimensional structures. Kidney [65], liver [66], stomach [67], and brain organoids [68] can show remarkable similarities to the corresponding tissues. Researchers have just begun to explore possibilities to model disease using organoids. Multisystem organoids have been developed, including intestinal organoids with components of the enteric nervous system [69]. Organoids may provide a means to produce more fully mature cells in vitro and may be useful for drug screening.

CHALLENGES AND FUTURE POSSIBILITIES IN DISEASE MODELING

It is difficult to mature cells fully in vitro, so it remains challenging to model late-onset diseases. Efforts have been made to age iPSC-derived cells artificially, such as progerin treatment [70] and telomere shortening [71], but it is not clear whether these treatments are realistic mimics of aging. Direct reprogramming from fibroblasts has been reported to preserve age-related characteristics of the donor [72], but direct reprogramming has shortcomings for disease modeling, as discussed earlier. However, in some cases, aspects of late-onset genetic diseases may be detected in iPSC-derived cells. This allows testing of drugs that can correct the cellular phenotype. In one example, ALS patient-derived motor neurons were found to have an abnormal electrophysiological phenotype, which could be corrected with a US Food and Drug Administration–approved anticonvulsant drug, Retigabine [73]. A clinical trial was initiated in 2016 (https://clinicaltrials.gov/ct2/show/NCT02450552) to evaluate the effects of Retigabine on motor neuron activity in people with ALS.

PERSONALIZED MEDICINE

For decades, drug discovery has relied heavily on a reductionist approach in which high-throughput screening is used to identify biologically active molecules based on expression of a recombinant target protein of interest in a single transformed cell line. The transformed lines used in these screening processes tend to be of similar ethnic backgrounds. Decades of experience using this model led to the finding that the genetic background of an individual can determine the success or adverse effects of a particular drug. For this reason, regulatory agencies recommend including a diverse patient population in later-stage clinical trials. A genetically diverse group of iPSC-derived cells would be instrumental in predicting these types of genetically dependent effects in the preclinical stages, making the drug development process more efficient [74].

Proof-of-concept toxicity studies have been performed in stem cell–derived cardiomyocytes [75], hepatocyte-like cells [76], and neurons [77]. However, to draw associations between genotype and drug response, as well as identify associated biomarkers, large collaborative initiatives are needed to generate cell banks that cover diverse ethnic groups, capturing a range of genotypes [74]. Carefully chosen sets of genetically diverse iPSCs could then be used for preclinical development.

In addition to the ethnicity of the patient, multiple disease mechanisms are likely to contribute to one disease phenotype, and one drug is not appropriate or sufficient for all cases. For example, cystic fibrosis is an autosomal recessive disease that is caused by mutations in a gene called cystic fibrosis transmembrane conductance regulator (CFTR), which encodes for a chloride channel. Mutations in this channel result in abnormal regulation of salt and

water, causing severe respiratory and gastrointestinal symptoms. More than 1900 CFTR mutations have been identified (www.genet.sickkids.on.ca). The type of mutation has a direct effect on the efficacy of commonly used medications. For example, for patients who carry a G551D mutation, CFTR channel activity is reduced. Ivacaftor, an oral agent and channel agonist designed to prolong the time in which the chloride channel is open, was effective in treating symptoms in this subgroup of patients [78]. However, this patient subgroup accounts for only 5% of patients with cystic fibrosis. For most patients, a truncation in the protein prohibits proper trafficking to the plasma membrane. In such cases, a channel agonist is ineffective. Another therapeutic, Lumacaftor, which aids in transporting CFTR to the cell surface, is more appropriate for these patients [79]. Identifying the disease mechanism for each CFTR variant could be enhanced by using patient-specific iPSC modeling.

iPSC model systems have been used to predict the clinical outcome of patients with specific mutations. One study examined a selective sodium channel blocker and its effect on inherited erythromelalgia using patient iPSC-derived sensory neurons, and could correlate the in vitro results to patient response and specific mutations [80]. As another example, a retrospective study identified patients who clinically experienced drug-induced QT prolongation upon administration of a nonselective β-blocker, sotalol. Cardiomyocytes derived from these patients' iPSCs recapitulated the patient response in vitro, demonstrating differential arrhythmias after sotalol treatment [81]. These studies demonstrate the exciting potential for using iPSCs to predict patient outcomes and to choose better from clinically available interventions.

CELL THERAPY

Cell therapy is a fast-developing field in modern medicine. Traditionally, cell therapy was limited to bone marrow cells and blood stem cells, but with the ability to generate clinically relevant cell types from stem cells, pluripotent stem cell–based cell therapies are being developed. A potential advantage of using iPSC-based therapies is that the cell line can be chosen to be patient-specific or a close immunological match. Autologous transplantation of iPSCs and their derivatives is expected to be tolerated by the immune system, and patients could benefit from avoiding immunosuppressant treatment required for allogenic transplantation. The creation of banks of iPSCs designed specifically to serve as a close immunological match to a large percentage of the population may also be feasible by deriving cells from individuals homozygous for the major human leukocyte antigen (HLA) markers. Such banks would reduce the time and cost needed to produce cells for therapy. The practicality of these banks depends on the number of cell lines needed to match the population served by the bank. In a country such as Japan, it is estimated that a bank of 50–100 carefully chosen iPSC lines could match 90% of the population [82]. In contrast, the genetic diversity of the populations of Europe and the United States would necessitate banks with orders of magnitude more cell lines, which makes the design more challenging and less feasible. The HLA-matched iPSC banks have not yet been tested in human trials, and there is concern that minor antigens could cause rejection even when there is a perfect major HLA match.

The expectation of immune tolerance of autologous iPSC-derived transplants, however, is brought into question by reports showing that transplantation of mouse iPSCs into syngeneic rodents led to immunogenic teratoma formation [83]. Subsequent studies using rodent models, including a humanized mouse model to reconstitute a human immune system, produced conflicting findings. Whereas some reports [84,85] found that autologous transplants were not immunogenic, others [86] found significant immune response upon autologous transplant of some types of iPSC-derived cells. The autologous immune response may be caused by aberrant antigen expression induced by differentiation in vitro and might be cell type–specific. For example, iPSC-derived smooth muscle cells showed aberrant antigen expression but iPSC-derived retinal pigment cells did not [86]. Therefore, even for autologous transplantation, preclinical studies should include analysis of potential immune response.

Besides the potential immunological advantages of iPSCs, any cell therapy shares the common challenge of properly delivering the cell product [87]. For most cases, delivery of therapeutic cell types must be targeted to a region of interest. Although intravenous injection is simple and easy, it often results in cell capture and the destruction of delivered cells in the lungs or the liver. Consider, for example, the case of myocardial infarction. A myocardial infarct results from the loss of blood flow to a region of the heart, typically resulting from an occluded vessel. The chronic effects of an infarct are physical remodeling of the heart muscle tissue that hypertrophies as the heart struggles to adapt to a loss of function. In this case, restoration of muscle function through cell replacement could stop the remodeling process and provide symptomatic relief to millions of people. Preclinical studies on rodent and nonhuman primate myocardial infarction models reported that transplantation of embryonic stem cell–derived cardiomyocytes can be effective to prevent further deterioration of cardiac function by remuscularization of

myocardial infarcts [88]. However, the heart is not a mechanically hospitable environment for cell delivery. Cells must be physically and electrically integrated in an appropriate manner to provide meaningful improvements and avoid potentially harmful side effects in humans.

In other areas of the body, such as the central nervous system (CNS), transplanted cells are less likely to escape into the bloodstream. The relative ease of delivery is in part why most pluripotent stem cell–based therapies have focused on the CNS. Several groups have developed cell replacement therapies to treat macular degeneration, which is caused by the loss of the retinal pigment epithelium (RPE). The first trial to use iPSCs was led by Dr. Masayo Takahashi in Japan; the first patient was transplanted with autologous iPSC-derived RPE cells, and there are plans to use the HLA-matched Japanese iPSC bank for further trials [89]. PD is another area of intense interest for a pluripotent stem cell–derived cell therapy. PD results from progressive loss of A9 dopaminergic (DA) neurons in the substantia nigra, and by the time the symptoms are diagnosed properly, over 50% of the neurons have already been lost. In 2011, a robust protocol mimicking neuronal development was reported that used activation of the sonic hedgehog and wnt pathways to obtain patterned midbrain progenitor cells [46]. This group further generated DA neurons whose activity could be optogenetically controlled and demonstrated that when transplanted into a rodent PD model, these neurons could integrate into host circuitry, secrete dopamine, and restore movement control [47]. Clinical trials are planned in multiple countries, including Japan, the United States, and Sweden. Some groups plan to use autologous iPSC-derived DA neurons, whereas in Japan, the bank of HLA-matched iPSCs will be used. Other groups using ESCs plan to use immunosuppression for at least 1 year to prevent graft rejection. A unique consortium of researchers (www.gforce-pd.com) who are all arranging to use a similar cell type will provide results that will guide further decisions about whether autologous therapy offers benefits over allogeneic transplants.

CONSERVATION OF ENDANGERED SPECIES

One of the least straightforward and most challenging applications of iPSCs is their potential use in wildlife conservation. One such example is the ongoing effort to save an endangered species, the northern white rhinoceros (NWR), which is on the brink of extinction owing to poaching and civil wars. The three remaining NWRs in the world, including one aging male and two females, all have reproductive issues that prohibit propagation of the species. NWR fibroblasts were first reprogrammed into iPSCs in 2011 [90], and a consortium of researchers met in 2015 to develop a plan to save the NWR, which includes differentiating NWR iPSCs into gametes to use for assisted reproduction [91]. This approach is possible only because of the foresight of researchers who, over the past 3 decades, preserved dermal fibroblasts from 12 genetically diverse NWR individuals (http://institute.sandiegozoo.org/). Since 2011, fibroblasts from several more NWRs have been reprogrammed; the technical hurdles now lie in producing functional gametes and successfully implanting fertilized embryos into surrogate hosts. It was reported that functional oocytes were generated entirely in vitro from mouse iPSCs, which provides further hope that the same can be done with endangered species iPSCs [92].

CONCLUSIONS AND FUTURE DIRECTIONS

For decades, human diseases were modeled in the mouse because of the genetic tools available to alter the mouse genome. hESCs began to be used to generate multiple human cell types that could be used for clinical cell therapy and disease modeling. However, hESCs lacked genomic diversity [93], and although there are hESC lines with genetic mutations, they cannot be linked to the phenotype of a living individual. Reprogramming and derivation of iPSCs have revolutionized the fields of stem cell biology, regenerative medicine, and the study of human disease. iPSCs share all of the benefits of hESCs and also have the advantages of genomic diversity.

The future of reprogramming is difficult to predict because the technology is developing so quickly. For example, one can imagine moving from the culture dish to in vivo reprogramming. Such an approach could be used to augment tissue-specific stem cells to enhance regeneration; however, improvements are necessary in the control over the delivery of the factors in vivo. Interestingly, a study investigated the short-term expression of the Yamanaka factors in genetically engineered rodents and reported a prolonged life span and decreased recovery time after injury [94]. Studies such as this open the door to potential therapies based on iPSC technology.

A complementary technology that is also evolving at a rapid pace is the CRISPR/Cas9 system and other genetic engineering methods that enable targeted genomic editing. Efficient genome editing of iPSCs can be used to correct mutations, allowing autologous cell therapy for genetic disease. Gene editing can also produce better models for disease, allowing the introduction of specific disease-related variants into iPSCs that can be used to better understand phenotypic expression of mutant and wild-type alleles. The applications of iPSCs are a new tool in 21st-century medicine that will improve our understanding of human disease and enable novel approaches to treat currently untreatable diseases.

List of Acronyms and Abbreviations

ALS Amyotrophic lateral sclerosis
CFTR Cystic fibrosis transmembrane conductance regulator
DA Dopaminergic
ESCs Embryonic stem cells
GWAS Genome-wide association study
hESCs Human embryonic stem cells
iPSCs Induced pluripotent stem cells
NWR Northern white rhinoceros
PD Parkinson disease
RPE Retinal pigment epithelium
SNCA α-Synuclein
TS Timothy syndrome

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CHAPTER

12

Multipotent Adult Progenitor Cells

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STEM CELLS

Stem cells have the unique capacity to self-renew, either symmetrically or asymmetrically. If stem cell divisions are symmetrical, the two descendant cells become stem cells, expanding the stem cell pool. However, postnatally, most stem cells divide asymmetrically, creating one stem cell and a differentiated cell or progenitor cells, resulting in maintenance of the stem cell pool [1]. Stem cells are unspecialized but can differentiate into multiple or all types of cells, depending on their potency. Totipotent stem cells present in the zygote or in the morula after a few divisions postfertilization can contribute to all of the cell types of embryonic development, including extraembryonic tissues such as, placenta, yolk sac, amnion, trophoblast, and extraembryonic endoderm lineages. Pluripotent stem cells (PSCs) are present in the inner cell mass (ICM) of the 4- to 6-day-old blastocyst. These pluripotent cells can also be derived from the ICM as embryonic stem cells (ESCs) [2–6]. Similar cells, induced PSCs (iPSCs), can be derived from adult somatic cells after overexpression of complements of transcription factors (TFs) such as *Oct4*, *Sox2*, *Klf4*, and *C-myc*. These pluripotent cells can differentiate into cells of three germ layers (ectoderm, mesoderm, and endoderm) in vitro as well as primordial germ cells [7,8] and extraembryonic endoderm [9]. When injected in vivo, they form teratomas, whereas when they are allowed to self-assemble in vitro, they form embryoid bodies.

Mouse ESCs (mESCs) are commonly cultured on feeders with leukemia inhibitory factor (LIF) and express cell surface marker SSEA1 [10,11] as well as *Oct4* [12], *Sox2* [13], *Nanog* [14], and *Rex1* [15]. Morula aggregation or tetraploid complementation studies showed that mESC contributes to chimera formation in the embryo proper or fetus, amnion, placenta, yolk sac mesoderm, trophectoderm, and germline cell types except for extraembryonic endoderm and [16,17]. Culture of mESCs in a so-called "naive" state, resembling cells in the ICM, has been accomplished by culture with inhibitors of glycogen synthase kinase 3β, extracellular signal-regulated kinase 1/2, and LIF [18].

Human ESCs (hESCs) are similar to mouse epiblast stem cells (mEpiSCs) and are established from postimplantation embryos. hESCs express SSEA4, TRA-1-60, and TRA-1-81 as well as the same TFs as are found in mESCs. As such, like mEpiSCs, hESCs do not depend on feeders or LIF, but on basic fibroblast growth factor and activin/nodal signaling to maintain pluripotency [19]. Multiple culture conditions and activation of specific TF gene networks has allowed the conversion of hESCs/iPSCs into a naive-like cell state, the direct derivation of naive PSC from blastocysts, and reprogramming of iPSCs from somatic cells [20–23]. Aside from their possible use in regenerative medicine, PSCs can be used to model cell-fate specification and disease.

ADULT STEM CELLS

Adult stem cells (ASCs), which include multipotent stem cells, can be defined as less or undifferentiated cells that are present with more highly differentiated cells in different organs. The primary role of ASCs is to maintain cell turnover and repair the tissue in which they reside. However, evidence suggests that they may be more versatile than previously thought and may be able to generate cell types different from cells of the tissue of origin. ASCs have been classified by some on the basis of the tissue source of origin. In 1906, Maximov postulated that bone marrow (BM) contains hematopoietic stem cells (HSCs). HSCs can be isolated from BM or mobilized peripheral

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blood. HSCs from BM or umbilical cord blood (UCB) can reconstitute all mature blood cell lineages after transplantation into irradiated BM of the animal [24]. Since that discovery, neural stem cells (NSCs) have been isolated from the subventricular zone (SVZ) and dentate gyrus of hippocampus of the adult brain [25,26] and the LGR5-positive stem cells from gastrointestinal tissues [27]. Hair-follicle bulge stem cells [28], limbal stem cells [29,30], epidermal stem cells [31] and MSC, among others, have also been isolated.

In 1974, Friedenstein and colleagues described the presence of fibroblast colony-forming cells in BM as part of the hematopoietic microenvironment or niche [32]. These were characterized by Caplan and Prockop and designated "mesenchymal stem cells" or "mesenchymal stromal cells" (MSCs) [33–36]. MSCs are clonogenic cells that can undergo multiple rounds of self-renewing cell divisions and differentiate into multiple mesodermal cell lineages, including adipocytes, chondrocytes, osteocytes, smooth muscle cells, fibroblast cells, and hematopoietic supportive "stromal" cells [32,33,36]. According to the International Society for Cellular Therapy, human MSCs are plastic-adherent and maintained under standard culture conditions. As much as 95% or more of MSC are positive for CD29, CD105 (SH2), CD106, CD73 (SH3), CD90, CD44, CD49a-f, CD166, Stro-1, CD271, and CD146, but they do not express hematopoietic markers such as CD34, CD45, CD14, CD11b, CD79α, CD19, or human leukocyte antigen (HLA) class II [37,38]. MSCs have proangiogenic, immunomodulatory properties [39] and can be expanded for multiple passages, which makes them highly interesting from a therapeutic perspective. MSCs can be derived from multiple tissues, including BM, adipose tissue [40], UCB [41], and placenta, among others [42].

Many reports have demonstrated that adherent cells from different tissues may display greater differentiation potential than that of MSCs. For instance, in 2002, Jiang et al. described novel culture conditions that allow the isolation of multipotent adult progenitor cells (MAPCs) [43]. Other such cells derived from BM were termed multipotent stem cells [44] and marrow isolated adult lineage inducible cells (MIAMI) [45]. Unrestricted somatic stem cells derived from placental cord blood have also been reported [46]. In addition, cells with apparent greater differentiation potential were derived from amniotic fluid [47], fetal liver (human fetal liver multipotent progenitor cells) [48], BM (very small embryonic-like cells) [49], human UCB [50], and BM, heart, and liver human multipotent adult stem cells [51]. In this chapter, we provide a brief update on the origin, isolation, and characterization and in vitro and in vivo differentiation potential of rodent MAPCs (rMAPCs) and human MAPCs (hMAPCs) and their use in clinical trials.

ISOLATION OF RODENT MULTIPOTENT ADULT PROGENITOR CELL

MAPCs from BM of mice, rats, and humans were first reported in 2001. rMAPCs were expandable in the long term in vitro without senescence. At the single-cell level, MAPC differentiated into mesoderm, endoderm, and ectoderm cell types. Furthermore, we demonstrated that when injected into the blastocyst, Rosa26 mouse-derived MAPC contributed to multiple somatic tissues of the mouse [43]. Murine MAPCs (mMAPCs) were also isolated from muscle and brain [52]. From 2005 to 2006, as described in Subramanian et al. we further optimized culture conditions for the robust isolation of rMAPCs [53]. The most important differences were that cells were maintained under 5% O_2 conditions and were subcloned at very low densities (5 cells/well of 96-well plate) for 6–8 weeks [43,53,54]. In this way, clones could be more easily isolated based on typical MAPC morphology and expression of *Oct4*, a TF, and the cell surface markers SSEA1 and CD31. High *Oct4* clones were more likely to have greater expansion potential in culture.

In 2007, we described the results of a comparative microarray transcriptome analysis of rMAPC isolated using the detailed method described in Subramananian et al. BM MSC, and ESC. This demonstrated that gene expression of the rMAPC differed significantly from MSC and ESC. rMAPCs expressed several ESC-specific genes including *Oct4*, *Rex1*, *Sall4*, *Rex1*, and *Esrrb* and a number of ESC-associated transcripts but did not express *Sox2* or *Nanog*. As noted, rMAPCs express primitive endoderm markers such as *Sox7*, *Sox17*, *Gata4*, *Gata6*, *Foxa2*, *Hnf1b,* and *Hnf4a* [55], similar to the nascent hypoblast of developing blastocysts [56].

In 2008, Debeb et al. described the isolation of rat extraembryonic endodermal precursor (rXENP) from rat blastocyst [57], which like rMAPC, express *Oct4* as well as *Sox7*, *Sox17*, *Gata4*, *Gata6*, *Foxa2*, *Hnf1b*, and *Hnf4a*. To address whether rMAPCs from BM correspond to rXENP derived directly from the rat blastocyst, Lo Nigro et al. assessed whether rat BM MAPC cultured in fibronectin in 2% fetal calf serum (FCS), LIF, platelet-derived growth factor (PDGF), and epidermal growth factor (EGF) could express rXENP characteristics by culturing on rat feeders and LIF. These studies demonstrated that when rMAPCs were cultured in rXENP culture conditions, the rMAPCs were capable of acquiring certain rXENP features, and vice versa. Moreover, the transcriptome and in vitro and

in vivo differentiation potential of rMAPC and rXENP cells were highly similar: They differentiated to mesoderm and endoderm in vitro and created yolk sac–like tumors in vivo.

In addition, these studies suggested that the rMAPC characteristics are affected in vitro during long-term culture conditions [58]. Related findings have been reported for spermatogonial stem cells [59–61] and epiblast stem cells [62], i.e., they can be reprogrammed into an ESC-like state by culturing the cells under ESC conditions. We also demonstrated that rMAPC culture conditions enabled the isolation of extraembryonic endoderm precursor cells from rat blastocyst within 2–10 days after plating blastocyst. Therefore, we demonstrated that the culture conditions favor the growth of rXENP cells. These culture condition characteristics may be important to induce the transdifferentiation of BM cells to this phenotype or allow the rapid expansion of cells that are spontaneously reprogrammed to this phenotype in BM cultures. Which of these two hypotheses is correct is not clear.

ISOLATION OF HUMAN MULTIPOTENT ADULT PROGENITOR CELLS

hMAPCs have also been isolated from human adult BM [63–65]. In 2011 [66], Roobrouck et al. demonstrated that hMAPCs can be expanded more than 70 population doublings, significantly more than hMSCs and human mesoangioblasts. In contrast to rMAPCs, hMAPCs were cultured without LIF; but similar to rMAPCs, the hMAPCs were cultured with FCS, EGF, and PDGF-BB and under hypoxic conditions. Phenotypically, the hMAPCs were positive for CD13, CD73, CD90, and CD105+; expressed low expression levels of HLA class-1 and CD146; and were negative for HLA-DR, CD140a, CD140b, the pericyte marker NG2, alkaline phosphatase, hematopoietic antigens such as CD34, CD45, and CKIT, as well as the endothelial antigens KDR, TIE2, VE-cadherin, intercellular adhesion molecule-1, and CD31 [66]. Comparative transcriptome studies demonstrated that hMAPCs differ from ESCs, monoclonal antibodies, and MSCs. Although quantitative reverse transcriptase–polymerase chain reaction identified *OCT4* transcripts in hMAPC, levels in hMAPCs were significantly lower than in rodent cells or hESCs [66,67]. Roobrouck et al. demonstrated that the expansion potential of the hMAPCs may be partly affected by the culture conditions used to isolate and maintain the cells. Indeed, when human BM MSCs were cultured under MAPC conditions, the expansion potential increased, and when hMAPCs were cultured under MSC conditions, the expansion potential decreased. This was also associated with a partial reversal in the transcriptome characteristics for MSCs and MAPCs, which demonstrated that the phenotype and transcriptome profile are partly imposed by cell culture conditions [66].

DIFFERENTIATION POTENTIAL OF RODENT AND HUMAN MULTIPOTENT ADULT PROGENITOR CELLS IN VITRO

rMAPCs and hMAPCs differentiate into mesodermal lineage cells such as osteogenic [68,69], chondrogenic [70], adipogenic [67,71], and smooth muscle [72,73]. In contrast to MSCs, rMAPCs and hMAPCs also differentiate into endothelial cells [74–76]. Unlike hMAPCs, rMAPCs differentiate into functional hepatocyte [77–79] and glucose-responsive, insulin-producing pancreatic β-like cells in vitro and rescue streptozotocin-induced diabetic mice [80]. For hMAPCs, induction of hepatocyte-specific transcripts and proteins can be induced using differentiation protocols similar to those used for rodent cells; however, this differentiation is less robust [81]. A more robust differentiation to endoderm cells can be obtained by overexpressing endodermal TFs in hMAPCs, which yields a population of so-called induced endodermal progenitor cells (iENDO cells) that can be expanded for at least 40 cell doublings and differentiate into hepatocyte and endocrine pancreatic-like cells in vitro and in vivo. However, iENDO progeny are not fully mature functionally (Article under revision).

REGENERATIVE CAPACITIES OF MULTIPOTENT ADULT PROGENITOR CELLS

Hematopoietic Reconstitution With Multipotent Adult Progenitor Cells

In 2007, Serafini et al. demonstrated that green fluorescent protein (GFP)-labeled transgenic mMAPCs injected into lethally irradiated mice could reconstitute the hematopoietic system in the primary recipients, albeit after a long time, with the creation of HSCs. Thus, although mMAPCs could contribute to hematopoiesis in primary recipients, a thousandfold more mMAPCs were required for efficient engraftment [82]. HSCs harvested from these primary mice could reconstitute the hematopoietic lineages of secondary and tertiary recipients with normal kinetics and protect them from lethal irradiation.

Immunomodulatory Properties of Low-Oct4 Murine Multipotent Adult Progenitor Cells and Human Multipotent Adult Progenitor Cells: In Vitro, Preclinical, and Clinical Studies

In Vitro Effects of Multipotent Adult Progenitor Cells on T Cells, B Cells, and Natural Killer Cells

In 2009, Highfill et al. demonstrated that high-Oct4 ($Oct4^{high}$) mMAPCs can suppress allogenic T-cell activation and proliferation [83]. This suppression occurred through prostaglandin E2 synthesis by MAPCs, which decreased proinflammatory cytokine production [83]. However, Luyckx et al. demonstrated that an $Oct4^{high}$ mMAPC clone had an immunostimulatory effect at a low stimulator to effector (S-E) ratio (100:1 and 10:1) in vitro, whereas a suppressive effect was found at an S:E ratio of 1:1 [84]. On the other hand, when $Oct4^{low}$ mMAPCs, MSCs, or ESCs were tested, the immunostimulatory effect observed with $Oct4^{high}$ mMAPCs was not seen. Furthermore, a local suppressive effect was found after $Oct4^{high}$ mMAPC and mMSC injection in a mouse model of graft versus host disease (GVHD) [85]. Although the exact mechanisms responsible for the differing effects of $Oct4^{high}$ and $Oct4^{low}$ mMAPCs in vitro are not fully understood, these experiments demonstrated that $Oct4^{high}$ and $Oct4^{low}$ mMAPCs have immunosuppressive effects in vivo if delivered locally.

In 2013, Reading et al. demonstrated that hMAPCs suppress T-cell proliferation and type 1 T helper (Th1) and Th17 cytokine production while increasing interleukin (IL)10 production. Furthermore, hMAPCs inhibited the proliferation of autoreactive T cells from patients with type 1 diabetes [86]. In 2015, the same group reported that culturing hMAPCs with CD4 T^+ cells and $CD14^+$ monocytes resulted in the suppression of IL7-dependent T-cell expansion and prevented Th1 (interferon [IFN]-gamma and tumor necrosis factor-4α), Th17 (IL17), and Th22 (IL22) proinflammatory cytokine production. Transwell studies demonstrated that soluble factors, including prostaglandin E, from MAPCs suppressed the T cells [87]. In 2016, Plessers et al. demonstrated that hMAPCs also blocked cytotoxic $CD8^+$ T lymphocytes [88].

Aside from affecting T-cell function, Jacobs et al. reported the effects of hMAPCs on natural killer (NK) cell function. hMAPCs express lower levels of class 1 MHC compared with the MSCs and express inhibitory signals for NK cells, such as poliovirus receptor and unique long−16 binding proteins-2/5/6. When resting NK cells were cocultured with allogeneic hMAPCs at effector−target ratios of 1:1 to 8:1 for 24 h, NK cells did not kill hMAPCs and blocked cytolytic functions of NK cells, because NK-sensitive K562 target cells were not killed. In contrast, IL 2−stimulated NK cells remained capable of killing hMAPCs. hMAPCs dose dependently reduced NK cell proliferation in an indoleamine 2,3-dioxygenase−dependent manner but did not influence the cytotoxic capacity of NK cells induced by IL-2, unless the hMAPCs were also activated using IFN-gamma. Thus, the mutual interaction between NK cells and hMAPCs depends on the activation state of NK cells and the priming of hMAPCs [89].

Finally, Ravanidis et al. demonstrated that rMAPCs can also suppress activated T-cell expansion, and that when challenged with proinflammatory cytokines, the rMAPCs produced chemokines as well as neurotrophic factors, including vascular epithelial growth factor (VEGF) and ciliary neurotropic factor, which could partly protect against hydrogen peroxide−induced death of the oligodendrocyte cell line (OLN93) [90].

In Vivo Immunodulatory Effects

Effect of Multipotent Adult Progenitor Cells on Graft Versus Host Disease

In the 2009 study described earlier, Highfill et al. demonstrated that when $Oct4^{high}$ mMAPCs were injected systemically, the cells did not home to sites for allopriming or suppress GVHD, but when they were injected locally in spleen at BM transplantation, mMAPCs suppressed T-cell proliferation and reduced GVHD via prostaglandin E2 synthesis [83,91]. Similarly, Luckx et al. demonstrated that although $Oct4^{high}$ mMAPCs suppressed local alloreactive T-cell expansion in lymph nodes in vivo, they failed to suppress GVHD when injected systemically [84]. By contrast, Kovacsovics-Bankowski et al. demonstrated that rat MAPCs were immunomodulatory, and when infused intravenously, they could reduce GVHD-related mortality in a rat model of GVHD [91].

In 2014, Maziarz et al. conducted a multicenter phase 1 dose escalation study to access the safety of clinical-grade MAPCs (MultiStem, Athersys, Inc., Cleveland, OH) in allogenic HSC transplantation. This study demonstrated that hMAPC infusions were tolerated without association of any known toxicity, graft failure, incidence, or infection. At the highest single dose (10 million cells/kg body weight) a reduced incidence of grade II to IV GVHD was seen 11.1% (one in nine patients) and no grade III and IV GVHD cases were observed compared with the lower single dose (1.5 million cells/kg body weight) in which 37% grade II to IV (37%) GVHD was observed [92]. Of note, no grade III or IV GVHD cases were observed in the highest single-dose cohort.

Effect of Multipotent Adult Progenitor Cells on Graft Survival When Cotransplanted With Other Cells

Cunha et al. demonstrated that cotransplantation of mouse pancreatic islets and an hMAPC composite pellet under the kidney capsule of alloxan-induced diabetic C57BL/6 mice improved islet function and glycemic control, secretion of c-peptide, and glucose tolerance compared with mice receiving islets alone or separate pellet transplantations of islets and hMAPCs. Moreover, hMAPC production of angiogenic growth factors including VEGF in vitro and in vivo is hypothesized to be responsible for improved graft neovascularization and enhanced islet function [93].

Role of Multipotent Adult Progenitor Cells as Immunomodulation in Solid Organ Transplantation

In 2012, Eggenhofer et al. demonstrated that infusion of third-party MAPCs in an allogenic rat model for heterotopic heart transplantation induced long-term allograft survival with low-dose pharmacological immunosuppression compared with animals treated with immunosuppressive drugs alone. Furthermore, hearts recovered from MAPCs treated animals could be successfully regrafted into naive animals with no immunosuppression [94]. Transcriptome studies demonstrated that the MAPC-induced tolerogenicity was caused by creating tolerogenic macrophages in the heart. Indeed, when monocytes and macrophages were depleted in the first recipient by intravenous administration of clodronate-filled liposomes in organ recipients before MAPC infusion, the tolerogenic effect of MAPCs was diminished and long-term acceptance of graft was reduced [94]. In a more recent study, the effect of clinical-grade hMAPC on the need for immunosuppression in clinical liver transplantation (Mesenchymal Stem Cells in Solid Organ Transplantation) was assessed in a phase I, dose escalation, safety, and feasibility study. Patients received one portal vein and one intravenous injection of third-party hMAPCs. Initial results indicate that no toxicity was found in MAPC-treated patients [95,96]. The clinical effect on liver rejection cannot yet be evaluated.

Multipotent Adult Progenitor Cell Immunodulatory and/or Trophic Effects in Ischemic Disease

Several preclinical and clinical studies in ischemic diseases have demonstrated a role for immunomodulatory and/or trophic effects that affect the degree of ischemic damage in the central nervous system, heart, and limbs.

Ischemic Stroke In 2010, Mays et al. demonstrated the neuroprotection of endogenous tissues at the site of injury and improved locomotor function when hMAPCs were injected directly into the striatum of a rat model of ischemic stroke. It was hypothesized that this was caused in part by the secretion of trophic factors by hMAPCs and reduced neuroinflammation at the site of injury. This resulted in decreased neuronal cell death and increased neoangiogenesis, indirectly contributing to tissue regeneration [97]. Similarly, Mora-Lee et al. in 2012 showed that when hMAPCs or MSCs were transplanted in an immunodeficient mouse model of stroke, animals treated with hMAPCs revealed a decreased loss of brain tissue that was associated with increased angiogenesis, and reduced inflammation and glial scar 28 days after stroke. In addition, increased proliferation of NSCs in the SVZ and survival of new neuroblasts were observed in hMAPC-treated animals [98].

In 2014, Hess et al. outlined the clinical protocol for a phase-1/2 randomized, double-blind, placebo-controlled, multicenter dose escalation trial in patients with ischemic stroke who were treated with MultiStem [99].

Jellema et al. demonstrated in a preclinical animal model for hypoxic-ischemic injury in fetal sheep that systemic administration of MAPCs reduced the duration of seizures, prevented microglial proliferation, and modulated peripheral inflammatory responses [100].

Spinal Cord Injury Multipotent adult progenitor cells and related cell types have been shown to modulate the inflammatory response after spinal cord injury, driving macrophages toward an alternatively activated phenotype and reducing the effects of the inhibitory glial scar [101,102]. DePaul et al. demonstrated that intravenous delivery of hMAPCs in an acute contusive spinal cord injury model reduced macrophage-mediated axonal cell death and decreased inflammation by homing MAPCs into the spleen. Functionally, improved recovery of locomotion and urinary function was documented that correlated with reduced macrophage infiltration and increased tissue sparing at the lesion site. Furthermore, the researchers reported a significant increase in arginase 1, a well-established marker of alternatively activated macrophages, in the acutely injured spinal cord. Biodistribution studies demonstrated that hMAPCs preferentially homed to the spleen. These results demonstrate that hMAPCs exert their primary effects in the periphery and support the hypothesis that hMAPCs alter the dynamics of the inflammatory response to central nervous system injury, ultimately leading to improved outcomes [103].

Traumatic Brain Injury In another study, intravenous injection of rMAPCs after traumatic brain injury (TBI) protected rats from neurovascular damage, also by interaction with resident splenocytes. This resulted in improved

preservation of the blood–brain barrier [104]. Intravenous rMAPC delivery after cortical injury induced T-regulatory cells and increased the M2/M1 macrophage ratio in splenocytes and plasma [105]. In addition, in vitro studies demonstrated that microglia activation was modulated by secreted factors from rMAPC–splenocyte cocultures, and these changes resulted from an increase in M1 macrophage apoptosis. Finally, it was shown that infusion of rMAPCs improved spatial learning in a TBI rodent model [106]. Therefore, it appears that neuroprotection in TBI (and possibly other brain or spinal cord injuries) is at least partly mediated by an interaction between MAPCs and splenocytes, which results in the modulation of activated microglia.

Ischemia–Reperfusion Injury The antiinflammatory properties of hMAPCs were also examined in an ex vivo lung explant model of ischemia–reperfusion injury [107]. The intratracheal administration of hMAPCs was shown to reduce cold-induced ischemia lung injury and inflammation and suggested that hMAPCs could be used ex vivo to reduce the effects of reperfusion after long-term cold storage of donor lungs.

Myocardial Infarct In 2007, Pelacho et al. demonstrated that direction injection of *Oct4*^{low} mMAPCs in the border zone surrounding a heart infarction in mice induced the functional improvement of left ventricular contractile cardiac function. The beneficial effect of MAPCs was mainly trophic, because no conversion of MAPCs to myocardial cells was observed, but improved host angiogenesis and changes in local inflammatory response were observed, which likely explained the improved cardiac function [108]. This study was further confirmed in a rat model in which syngeneic and allogeneic rMAPCs were tested in a rat model of acute myocardial infarction (MI) [109] and in the setting of xenogeneic delivery of hMAPCs in a mouse MI model by Dimomeletis et al. in 2010, even though authors suggest that MAPC conversion to cardiomyocytes was seen, what other teams were less conclusive about [110]. Similar observations were found by Zeng et al. [111], when swine MAPCs were directly injected into the heart of allogeneic swine, without or with immunosuppression. This initial study was followed by a longer-term assessment study in which the same group demonstrated that MAPCs imparted long-term functional and bioenergetic improvement in a swine MI model [112]. These studies formed the basis for a phase I clinical trial in which allogeneic clinical-grade hMAPCs (MultiStem) were administered by adventitial delivery into the coronary artery in acute MI patients with patients with an ejection fraction of 45% or less. The results demonstrated that the cells were well-tolerated and that the ejection fraction 4 months posttherapy was increased by ±10% in groups that received 50 or 100 million MultiStem, which was in the 50 and 100 million group also accompanied by a 25% and 8% increase in left ventricular stroke volume, respectively [113].

Peripheral Hind Limb Ischemia Aranguren et al. in 2008 compared the effect of grafting unselected murine bone marrow cells (mBMCs), *Oct4*-expressing mMAPCs, or *Oct4*-expressing mMAPCs predifferentiated to vascular progenitors (mMAPC-VP) in a mouse model of moderate ischemic limb. They demonstrated that mMAPCs, in contrast to mBMC and mMAPC-VP cells, resulted in a sustained improvement in muscle regeneration and function. In addition, when tested in a much more severe model of limb ischemia, they demonstrated that both undifferentiated *Oct4*^{high} mMAPCs and hMAPCs remedied vascular and muscular deficiencies [114]. In a subsequent study, in which human angiogenic progenitor cells isolated from BM based on the expression of AC133 cells and hMAPC cells were compared, hMAPCs improved angiogenesis and muscle regeneration and function significantly better than AC133 cells. These studies demonstrated that the largest effect of mMAPC/hMAPC was through the production of trophic proangiogenic and regenerative stimuli.

Antitumor Effects of Multipotent Adult Progenitor Cells in Glioma

Oct4^{low} mMAPC syngeneic expressing enhanced GFP and firefly luciferase-herpes simplex virus thymidine kinase and labeled with superparamagnetic iron oxide nanoparticles labeled (1% or 10%) were grafted in glioblastoma (GL261)-bearing animals. Magnetic resonance imaging of the animals demonstrated that mOct4^{-} MAPCs were located near the glioblastoma and upon treatment with ganciclovir, a reduction was seen in tumor volume along with a better survival rate compared with control untreated animals. The antitumor effect was hypothesized to be at least partly mediated by the synergistic effects of the immunomodulatory properties of mMAPCs and suicide gene therapy [115].

Possible Mechanisms of Trophic Effects: Secreted Proteome of Multipotent Adult Progenitor Cells

In 2013, using a proteomics approach, Burrows et al. identified the secretome of hMAPCs over 72 h in vitro under steady-state conditions and in response to different inflammatory signals, specifically IFN-gamma or lipopolysaccharide (LPS), or the tolerogenic CD74 ligand, RTL 1000. These studies revealed that MAPCs secrete multiple

molecules involved in regulating extracellular matrix components, as well as chemokines, cytokines, and multiple molecules that affect angiogenesis, activation of growth factors, and the innate immune system. The secretome was highly differentially affected by incubating MAPCs with IFN-gamma, LPS, or RTL-100 [116]. These findings are in line with the immunomodulatory, protective, and proangiogenic effects of MAPCs in different disease settings; the precise contribution of the secreted factors identified in this proteome study will need further validation.

CONCLUSION AND FUTURE DIRECTIONS

We here describe MAPCs derived from rodents (mice and rats) and humans. Although both cell types initially were named MAPCs, based on the fact that they were isolated from BM of rodents and humans and had differentiation and expansion potential greater than MSCs, more recent studies demonstrated specific and important differences between rMAPCs and hMAPCs. Although rMAPCs express the pluripotency gene *Oct4* at levels near those in ESCs, have the potential to differentiate robustly into endodermal cells, and develop into yolk sac tumors when grafted in vivo, these findings do not hold true for human cells.

hMAPC that do not cause tumor formation have been tested extensively for their possible immunomodulatory and trophic effects in different animal models of transplantation, GVHD, central nervous system injury and ischemia, and cardiac and peripheral limb ischemia. The results from these preclinical studies are being tested in ongoing clinical studies using the clinical-grade MultiStem product.

Conflict of Interest Statement

Catherine M. Verfaillie is a consultant to ReGenesys, Leuven, Belgium.

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CHAPTER

13

Hematopoietic Stem Cell Properties, Markers, and Therapeutics

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INTRODUCTION

The hematopoietic system provides for the regulated production of the complete complement of mature blood cells in the peripheral circulation, which includes neutrophils, eosinophils, basophils, monocytes, lymphocytes megakaryocytes (platelets), and erythrocytes. These mature blood cells have a limited life span, which ranges from hours for granulocytes to weeks for erythrocytes and years for some lymphocytes. To maintain equilibrium in the hematopoietic system, the continual production of mature blood cells is required. The hematopoietic system is a self-renewal system in which hematopoietic stem cells divide and differentiate to produce maturing progeny as well as self-renew to maintain a pool of stem cells for the life of the individual. This chapter will define the properties of the hematopoietic system and review the current therapeutic uses of hematopoietic stem cells.

HEMATOPOIETIC STEM CELL PROPERTIES

Hematopoietic cells appear early in embryonic development. The first observable site of hematopoiesis in the mouse is the formation of blood islands in the yolk sac at 7.25–7.5 days postfertilization [1]. Controversy has surrounded the origin of hematopoietic stem cells during embryological development. In studies using parabiosed chorioamnionic membranes from two chick eggs of different sexes, it was determined that spleen, bone marrow, and bursa of Fabricius contained hematopoietic cells of the opposite sex [2]. The results suggested that the hematopoietic organs were seeded by stem cells circulating in the blood. When 7-day-old yolk sac cells were injected into irradiated chicken embryos, both myeloid and lymphoid tissues of the irradiated embryos were repopulated by the injected yolk sac cells, which suggested that the circulating cells were pluripotent, seeded the different organs, and differentiated [3]. Using a mouse model, similar studies demonstrated that yolk sac contained both stem and progenitor cell populations [1]. Yolk sac cells from 7- to 13-day old mouse embryos were injected into irradiated recipients to measure colony-forming unit–spleen (CFU-s) content. Yolk sac–derived CFU-s were detectable at day 8 of gestation and reached a maximum at day 11. These cells also contained hematopoietic progenitor cells, as measured by an in vitro colony assay. Neither hematopoietic stem nor progenitor cells were detectable by day 13 of gestation. The pluripotent nature of yolk sac cells was demonstrated by repopulation of both lymphoid and myeloid tissues of lethally irradiated recipients. The authors concluded from these studies that hematopoietic tissues in embryos were colonized by circulating stem cells that were derived from the yolk sac and that the yolk sac was the only de novo site of hematopoietic stem cell production [1].

In contrast, studies using chick–quail chimeras, in which 2-day-old quail embryos were grafted onto developmentally compatible chick yolk sacs, demonstrated that yolk sac blood island cells did not contribute to intraembryonic hematopoiesis [4]. Only quail hematopoietic cells were found in the thymus and spleen of the grafted quail embryos. The discrepancy in the data was explained by the gestational age of the embryos at which the yolk sac was examined. Seven-day-old chick embryo yolk sac–derived cells were used to repopulate the irradiated

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chick embryos [3]. Vascularization was established before day 7 of gestation in the chicken embryos; therefore, it was speculated that cell migration had resulted in the presence of intraembryonic-derived stem cells in the yolk sac before the gestational age of 7 days [4]. Another study demonstrated that hematopoietic cells that were able to colonize the thymus were present in the blood of 4-day-old chick embryos [5].

Lymphoid progenitor cells were shown to be derived from intraembryonic sites and not from yolk sac in a chick–chick chimera model [6]. Using a chick–quail chimera model, the intraembryonic source of hematopoietic stem cells was confirmed and the colonization of embryonic hematopoietic organs was shown to occur via interstitial migration of stem cells rather than the vascular seeding of the stem cells from the yolk sac [7]. To define the location of the hematopoietic stem cells further, anterior and posterior regions of quail blastoderms were grafted onto chicken blastoderms. The origin of the hematopoietic cells was localized to the posterior section of the grafted quail blastoderm; no hematopoietic stem cells were found in the cephalic or anterior grafted blastoderm [7]. The specific site of the origin of hematopoietic stem cells was not identified. However, it was speculated that colonization of the embryonic hematopoietic organs was from stem cells derived from adjacent tissues [7]. The dorsal aorta region of the chicken embryo was suggested to be the site of origin of hematopoietic stem cells [4].

The use of an amphibian developmental model also demonstrated an intraembryonic origin of hematopoietic stem cells [8]. Using an amphibian model containing cytogenetically unique cells, the study showed that there were two sites of hematopoiesis during early amphibian development: one from the ventral blood island mesoderm, which produced a transient population of erythrocytes, and the other from the lateral plate mesoderm in the region of the dorsal aorta-gonad-mesonephros (AGM) region, which produced definitive hematopoiesis [9]. The migration of hematopoietic precursors in the lateral plate was shown to be from the posterior to the more anterior pronephros and dorsal aorta [10].

The two distinct sites of hematopoiesis described in the amphibians may also apply to avian and mammalian hematopoiesis in embryo development. Erythroid cells derived from chicken yolk sac were able to colonize spleens of grafted quail embryos, but only transiently [11]. Chicken-derived erythroid cells were found in the spleen of grafted quail embryos between days 10 and 12; however, by day 13, only quail erythroid cells were seen in the spleen. In addition, up to day 10, chicken red blood cells were most of the circulating red blood cells; however, by day 13, up to 80% of the circulating red blood cells were derived from quail hematopoietic cells [11]. In a mouse model, 9.5-day-old mouse yolk sac cultured in vitro produced primitive erythropoiesis for only a short time [12]. However, if the yolk sac was cultured in a transmembrane organ culture in the presence of embryonic liver rudiment, the yolk sac developed into definitive erythropoiesis [12]. Definitive erythropoiesis was established whether or not there was direct cell–cell contact between the yolk sac and liver rudiment. This result indicates a role for the microenvironment and short-range factors in the induction of definitive erythropoiesis.

Placental Hematopoiesis

During fetal development, the placenta serves as the tissue responsible for maternofetal exchange as well as a source of cytokines and hormones [13]. Early studies in the mouse suggested that the placenta contains hematopoietic activity [14,15]. Hematopoietic cells are found in the placenta as early as day 9 of gestation, which suggests that the placenta may be a site of hematopoietic stem cell generation in situ [16]. However, the placenta may also be seeded with hematopoietic stem cells from the AGM region [17]. Hematopoietic stem cells are rapidly expanded in the placenta between days 11.5 and 12.5 of gestation and decline after day 13.5 of gestation [18]. The placenta may be a source of hematopoietic stem cells for the fetal liver via the fetal circulation [17].

Fetal Liver Hematopoiesis

The fetal liver is the primary hematopoietic organ for fetal development and is the primary site for expansion and maturation of fetal hematopoietic stem cells. Hematopoiesis in the fetal liver depends on the migration of hematopoietic precursor cells and is not a site for the de novo generation of hematopoietic stem cells. In the mouse, yolk sac erythropoiesis declines rapidly from day 15 of gestation with the switch of hematopoiesis to the fetal liver. By day 9.5–10 of gestation, the fetal liver is colonized by circulating hematopoietic progenitors [19]. During this early time of colonization, the major cell type is definitive erythroid cells probably representing progenitors originating in the yolk sac [20]. Lymphoid and myeloid lineages develop later. The first hematopoietic stem cells appear around day 11.5 of gestation and undergo extensive expansion in the fetal liver [21]. The yolk sac, AGM region, and placenta are the potential sites of origin for the cells seeding the fetal liver. Hematopoietic stem cells found in the fetal liver are

pluripotent, as evidenced by the repopulation of lymphoid and myeloid linages after fetal liver cell transplantation into lethally irradiated mice [22]. Using the CFU-s assay, the number of hematopoietic stem cells in the liver reached a maximum at day 18 of gestation and then fell sharply at birth [23]. No hematopoietic stem cells could be detected in the liver 8 days after birth [23]. Developmental changes in the fetal liver microenvironment may influence the homing, retention, support, and differentiation of hematopoietic cells [24]. In adult animals, under normal physiological conditions, liver extramedullary hematopoiesis does not occur. However, under severe physiological stress, extramedullary hematopoiesis can occur [25].

Bone Marrow Hematopoiesis

In adults, the major site of hematopoiesis is the bone marrow. The structure of bone marrow is not random. There is an ordered arrangement of hematopoietic stem and progenitor cells in relation to the hematopoietic microenvironment. Morphologically, the mouse femoral bone marrow consists of cords of highly vascularized hematopoietic tissue that is composed of structural microenvironmental cells that support the maintenance, proliferation, and differentiation of hematopoietic stem and progenitor cells. Spatial organization is important to the regulation of hematopoietic cells. The stromal component of the bone marrow microenvironment is composed of osteoblasts, fibroblasts, adipocytes, chondrocytes, and vascular cells [26]. Bone marrow stromal cells make up the supportive microenvironment for the maintenance of hematopoiesis through the production of growth factors, chemokines, and extracellular matrix, as well as direct cell—cell interaction.

A concept for a bone marrow niche was first proposed by Schofield [27]. He postulated that primitive hematopoietic stem cells reside in the niche in association with stromal cells. When a hematopoietic stem cell divides, one daughter cells remains in the niche representing self-renewal and the other daughter cells leaves the niche to undergo further proliferation and differentiation.

Two types of bone marrow niches have been identified: osteoblastic and vascular. The osteoblastic niche is located near the endosteal surface of the marrow and was first suggested from homing studies of transplanted hematopoietic stem cells [28]. Osteoblasts and bone marrow stromal cells produce SDF-1, which is a homing chemokine for hematopoietic stem cells [29]. The osteoblastic niche provides an environment that supports stem cell quiescence and the long-term survival of hematopoietic stem cells [30]. The vascular niche is located in the central medullary region of the bone marrow. Stem cells located in the vascular niche have access to circulating factors that may allow mobilization into the peripheral circulation via transendothelial migration [31].

In Vitro Hematopoiesis

An in vitro bone marrow culture system was developed by Dexter and colleagues; it supports the long-term maintenance of hematopoietic stem cells [32]. The adherent layer contains several stromal cell populations and is obligatory for the long-term support and production of stem cells [33]. Upon morphological examination of the adherent layer of long-term bone marrow cultures, primitive hematopoietic cells can be seen in close association with the stromal cells (Fig. 13.1 and 13.2).

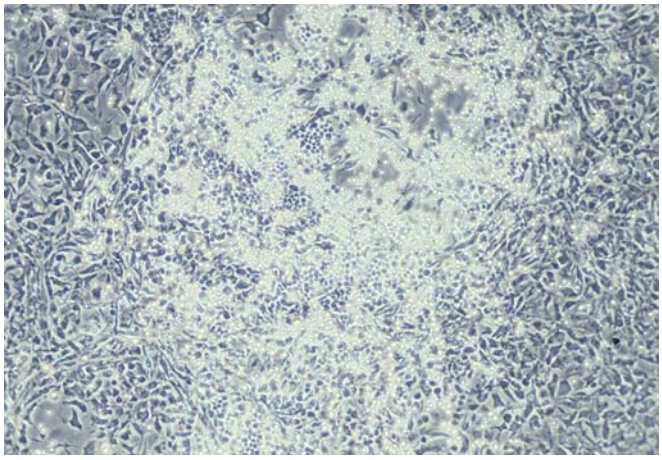

FIGURE 13.1 Photomicrograph of a long-term bone marrow culture showing the adherent stromal layer that contains bone marrow stromal cells and the associated hematopoietic cells (magnification, 20×).

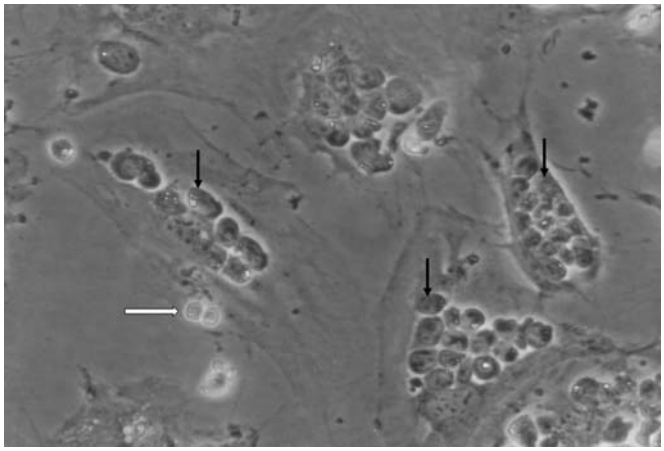

FIGURE 13.2 Photomicrograph showing the interaction of hematopoietic progenitor cells with bone marrow stromal cells. The *black arrows* point out the close association of the hematopoietic progenitor cells with the adherent stromal cells. This interaction is the "cobblestone area." The phase dark cells are hematopoietic progenitor cells that have migrated under the stromal cell (*black arrows*). The phase light hematopoietic progenitor cells are on top of the adherent stromal cell (*white arrow*) (magnification, 100×).

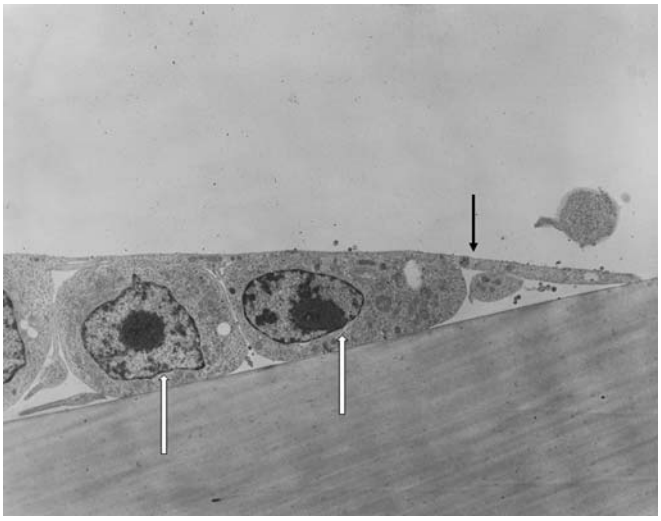

FIGURE 13.3 Transmission electron micrograph of a cobblestone area. Hematopoietic progenitor cells (*white arrows*) have migrated under the stromal cell (*black arrow*) and are in close contact with the stromal cell (magnification, 5550×).

These areas consist of multilayer cellular complexes that are hematopoietically active (Fig. 13.3).

Primitive CFU-s reside in the adherent layer, where they undergo proliferation and differentiation with some of the CFU-s, and many mature cells or their immediate precursors are released into the supernatant [34,35]. The adherent layer contains a larger proportion of more primitive (day 12) CFU-s and the nonadherent or supernatant cells contain a higher number of more mature (day 8) CFU-s [36,37]. Cell-to-cell contact appears to be essential in the in vitro maintenance of these stem cells. The development of in vitro long-term bone marrow cultures has been important in characterizing hematopoietic stem cells and their interaction with stromal cells.

Phenotypic Properties of Hematopoietic Stem Cells

Hematopoietic stem cells represent approximately 0.01% of nucleated cells within adult bone marrow. The identification and isolation of hematopoietic stem cells have been greatly enhanced by technical advancements in flow cytometry and the development of monoclonal antibodies. One of the first comprehensive phenotypic enrichments of mouse hematopoietic stem cells was reported in 1988 by Weissman and colleagues [38]. The procedure was modified over the years, but basically it involved the use of a cocktail of antibodies to deplete mature hematopoietic cells

with the resultant enrichment of hematopoietic stem and progenitor cells. The depletion cocktail included antibodies to B cells (CD45R/B220), granulocytes (Gr-1), macrophages (Mac-1), erythrocytes (Ter-119), and T cells (CD-4 and CD8). The resultant cell population was termed lineage negative (lin^-). Other antibodies were used to subdivide the enriched cell population further, including Sca-1 and c-Kit [39]. The $lin^-/Sca\text{-}1^+/c\text{-}Kit^+$ (LSK) cells represent approximately 0.05% of nucleated bone marrow cells [40]. Thy-1.1 and Thy-1.2 antibodies were also used; however, the expression of Thy-1.1 is mouse strain dependent, and mouse strains expressing Thy-1.2 show variability in expression; therefore, these two markers are not as useful in hematopoietic stem cell purification [41]. CD34 can also be used to subdivide the LSK population. The $CD34^-$ cells contain a more primitive stem cells than do the $CD34^+$. Therefore, long-term repopulating cells fall within the $LSK/CD34^-$ population. Morrison and colleagues used the signaling lymphocyte activation molecule family of cell surface markers to identify a long-term repopulating hematopoietic cell population ($CD150^+/CD244^-/CD48^-$) [31]. CD150 can be added to the LSK scheme to enrich hematopoietic stem cells further.

Another approach to identifying hematopoietic stem cells is the use of fluorescent dye exclusion rather than cell surface markers. This approach uses the stem cell property of the high expression of multidrug resistance (MDR) pumps, which remove drugs and dye from the cells, resulting in low fluorescence. Two different dyes have been used. Rhodamine 123 (Rho123) is a mitochondrial-specific dye. $Rho123^{lo}$-expressing bone marrow cells are enriched in long-term repopulating cells [42,43]. Use of the second dye was describe by Goodell and colleagues [44]. Hoechst 33,342 is a vital dye that binds to DNA; when it is excited by a UV laser, it emits at two wavelengths, 450 nm (Hoechst blue) and 675 nm (Hoechst red). Hematopoietic stem cells efflux the dye owing to their high expression of MDR pumps, resulting in a low concentration of fluorescence. Analysis of the Hoechst red versus Hoechst blue emissions reveals a unique plot with the stem cells present on the side of the fluorescent profile; therefore, it is termed the side population (SP). SP cells are enriched for hematopoietic stem cell activity; most of the hematopoietic repopulating ability is located in the lower portion of the SP fraction [45,46]. Dye exclusion assays can be combined with immunophenotyping methods to provide a more stringent selection of hematopoietic stem cells.

HEMATOPOIETIC STEM CELL THERAPIES

Bone Marrow Transplantation

During the production of nuclear weapons in the 1940s, it was discovered that the hematopoietic system was one of the more sensitive tissues to the effects of irradiation. Many investigators searched for methods to protect the hematopoietic system from the detrimental effects of irradiation. It was found that shielding the spleen would allow irradiated mice to survive [47]. In addition, the infusion of spleen cells also protected mice from lethal irradiation by reconstituting the hematopoietic system [48]. Injection of bone marrow cells also resulted in the recovery of the hematopoietic system and the survival of lethal irradiated animals [49]. During these studies, the dogma was that humoral factors produced by spleen and bone marrow were responsible for the recovery of hematopoiesis in the irradiated animals [50]. However, several studies in the mid-1950s demonstrated that hematopoietic protection after irradiation resulted from infused donor cells that contributed to hematopoietic recovery in the host [51–54]. This discovery of a cellular role in hematopoietic recovery from lethal irradiation initiated the age of hematopoietic stem cell transplantation for malignant diseases.

In 1957, Thomas and colleagues performed the first allogeneic transplantations on six patients who experienced various types of terminal illnesses [55]. The infusion of donor bone marrow cells demonstrated that this procedure could be administered safely; however, no lasting engraftment was noted owing to the modest levels of irradiation and chemotherapy given to the patients. Three years later, Thomas and colleagues transplanted two pediatric patients who had acute leukemia with syngeneic bone marrow after chemotherapy and total body irradiation [56]. Each patient had a twin sibling who provided the donor bone marrow. Although both patients experienced relapse of the disease several months after transplantation, the procedure resulted in hematopoietic reconstitution of the patients. Around the same time, Mathé and colleagues infused allogeneic bone marrow into people who had accidentally been exposed to radiation. Four of the five transplanted patients survived; however, the transplant recipients experienced only transient chimerism. Because the exact dose of irradiation that each victim received was impossible to determine accurately, they probably did not experience a lethal dose of irradiation, and survived owing to short-term hematopoietic support from the infused allogeneic cells and long-term autologous reconstitution of the hematopoietic system [57,58]. These early transplantations established the safety of bone marrow cell infusion, which led to wider use of allogeneic bone marrow transplants in the late 1950s and early 1960s. Unfortunately, no long-term

survivors were noted [59]. Many reasons contributed to this outcome, including the lack of human leukocyte antigen (HLA) matching, insufficient immunosuppression, and the lack of effective supportive care [60–62].

These early transplantation failures reduced enthusiasm for further attempts at clinical transplantation. However, animal studies continued with significant advances in immunosuppression and histocompatibility determination. These addressed issues with graft rejection involving the host immune system attacking infused donor cells, resulting in graft failure and donor-reactive T cells attacking the host tissues and organs such as skin, liver, and intestine and resulting in graft versus host disease (GVHD) [63–66]. Approximately 50% of patients with allogeneic transplantation experience GVHD even though they received major histocompatibility complex–matched donor cells and immunosuppressive drug treatment [67,68]. Other factors determining the development and severity of GVHD are recipient age, the conditioning regimen, and the source of hematopoietic stem cells [69].

Because of the significant morbidity and mortality associated with GVHD and the recognition of the role of donor T cells in GVHD, many clinical investigators examined T-cell depletion to reduce GVHD. Procedures to enrich for hematopoietic stem cells also resulted in donor T-cell depletion and, more important, reduced the number of tumor cells in the graft. However, it was found that patients who received T-depleted or hematopoietic stem cell–enriched grafts experienced an increase in graft failure, tumor relapse, and infection [70,71]. During this time, preclinical and clinical data showed that allogeneic transplantation provided an additional benefit, a graft versus tumor (GVT) effect. In a mouse model, Barnes et al. were the first to demonstrated the GVT effect. T cells within the donor graft were able to recognize and kill the host's tumor cells [72,73]. This effect occurred only in allogeneic, not syngeneic transplantations. Patients who developed GVHD experienced less tumor relapse; therefore, there was a link between GVHD and GVT effects [74–76].

Significant efforts have been reported to enhance GVT effects and suppress GVHD. One approach is to target minor histocompatibility antigens [77,78]. Another approach is to use regulator T cells to modify GVHD and maintain GVT effects [79] as well to use natural killer (NK) cells to augment GVT effects while circumventing GVHD [80,81]. In addition, the infusion of donor leukocytes after allogeneic transplantation was shown to induce remission in patients with chronic myelogenous leukemia [82]. The reduction in relapse in some patients with chronic myeloid leukemia was associated with GVHD; however, other patients achieved remission without GVHD.

Autologous Peripheral Blood Stem Cell Transplantation

During the 1980s and 1990s, clinical transplantation rapidly increased. In earlier times, bone marrow harvested from the posterior iliac crest or the sternum was the primary source of hematopoietic stem cells used in transplantation. However, other sources of hematopoietic stem cells were identified, including peripheral blood and umbilical cord blood.

In 1979, patients with chronic granulocytic leukemia (CGL) were successfully transplanted with autologous peripheral blood stem cells (PBSCs) collected in the chronic phase of the disease [83]. When the patients advanced to the acute phase of CGL, they underwent high-dose therapy and were transplanted with the cryopreserved PBSCs. The patients engrafted and returned to the chronic phase of the disease. This report demonstrated that CGL PBSCs could restore hematopoietic function in the patients with CGL. There were two reports in 1979 and 1980 in which syngeneic PBSC transplants failed [84,85], which reinforced concerns regarding the quality of circulating stem cells and their ability to reestablish bone marrow hematopoiesis [86]. The reason for failure of the PBSC transplants was not clear, but it may have been associated with the method of infusing the cells, which occurred over 1–2 weeks. In 1986, multiple centers reported successful transplantations using nonmobilized (steady-state) PBSC [87–90]. The number of hematopoietic stem cells was significantly lower in the peripheral circulation than in the bone marrow (0.06% and 1.1% $CD34^+$, respectively). Therefore, mobilization procedures were developed to increase the number of circulating hematopoietic stem and progenitor cells. One method was to collect peripheral blood after hematopoietic recovery, after nonmyeloablative chemotherapy. The myelosuppressive effects of chemotherapy induced a hematopoietic rebound, resulting in an increase in circulating hematopoietic stem and progenitor cells in the peripheral blood [91]. The timing of the hematopoietic rebound in the peripheral circulation was difficult to determine; therefore, this technique was not used extensively. After the development of recombinant cytokines, colony-stimulating factors were approved for clinical use. Granulocyte colony-stimulating factor (G-CSF) has become the preferred method of mobilization. PBSCs have since replaced the use of bone marrow stem cells in many transplant centers; approximately 75% of unrelated allogeneic hematopoietic stem cells transplantations and almost 100% autologous transplantations have been performed [92].

Allogeneic Peripheral Blood Stem Cell Transplantation

Initially, there was a reluctance to use allogeneic PBSC for transplantation because of the potential for an increase in GVHD. PBSC collections contain an approximately 10-fold greater number of T cells than a bone marrow harvest. The first reported use of allogeneic peripheral stem cells transplantation was in 1989 [93]. A patient with acute lymphocytic leukemia received a steady-state (nonmobilized) PBSC transplantation from an HLA-matched sibling. The donor preferred peripheral blood collection rather than undergoing a bone marrow harvest. The collections were T-cell depleted; however, the last collection was used unmanipulated to contain the estimated number of T cells in a bone marrow harvest. On day 27 posttransplant, a biopsy showed trilineage engraftment; however, long-term engraftment was not determined because the patient died on day 32 after transplantation. By the mid-1990s, several transplant centers reported successful allogeneic PBSC transplantations [94–96].

The reasons for the switch from bone marrow to PBSC transplantations were numerous. Bone marrow harvest requires general anesthesia with its associated risks and postharvest pain, and patients have reported fatigue [97]. PBSCs are mobilized using a 4- to 5-day course of cytokines (primarily G-CSF) and then isolated using apheresis in 1- to 3-day collection sessions, usually in an outpatient setting. The only requirement is venous access. Therefore, the ease of collection and lower incidences of adverse events may have been the driving force for the switch to PBSCs [98]. In addition, the number of hematopoietic stem cells in cytokine-mobilized PBSC collections was higher than in bone marrow harvests. This higher number of stem cells may be responsible for the more rapid hematopoietic engraftment and immune reconstitution associated with PBSC transplants compared with bone marrow transplantations [99,100]. PBSC transplantations are also associated with a decreased rate of relapse, which may be related to the higher number of T cells present in the PBSC collections and the subsequent increase in GVT effects compared with bone marrow harvests. However, there are some risks associated with PBSC transplantations. A small number of PBSC donors undergoing cytokine mobilization experience bone pain. Of more concern is spleen rupture [101]. With the increase in the number of T cells in the PBSC, an increase in the incidence of chronic GVHD has been noted with no increase in acute GVHD [102,103]. However, the higher chronic GVHD seen in PBSC transplant recipients compared with bone marrow recipients did not lead to an increase in mortality for the PBSC graft recipients [103]. Overall, the risk for disease relapse is lower in PBSC transplantation patients; however, the progression-free survival appears to be similar between PBSC and bone marrow transplant recipients [99,104,105].

Cord Blood Transplantation

The first report of the potential of cord blood as a source for hematopoietic transplantation occurred in 1972 [106]. However, it was the critical work by Broxmeyer that advanced cord blood from the bench to the bedside [107,108]. The first cord blood transplant was performed in 1988 for a 5-year-old patient with Fanconi anemia [109]. The donor cord blood cells were collected from a sibling and cryopreserved. After transplantation, the patient engrafted by day 22 and expressed donor chimerism. This success led to an expansion of the use of cord blood for allogeneic transplantation. Cord blood has also been used for unrelated transplantation in pediatric and adult patients who did not have a related donor [110,111].

Cord blood is a readily available source of allogenic hematopoietic stem cells that can be collected at the time of birth. Both public and private cord blood donor sites are available [112,113].

Challenges in using cord blood for transplantation include delayed engraftment, which may increase the risk for infection [114], and small volumes of cord blood collections, which limit the number of hematopoietic stem cells for engraftment of adults or large pediatric patients [115]. To overcome these challenges, two cord blood collections have been used. Double cord blood transplantations increase the cost and have been associated with an increased incidence of GVHD [116]. Another method for increasing stem cell numbers in cord blood collections is to use ex vivo expansion [117]. No significant improvement was seen in survival or transplantation outcomes with expansion; however, neutrophil engraftment was augmented.

Cord blood transplantation is a valid treatment for both pediatric and adult patients who do not have a matched sibling or unrelated donor. In the future, cord blood needs to be compared with other allogeneic donor sources to define more clearly the advantages of cord blood transplantations.

Hematopoietic Stem Cell Transplantation for Severe Combined Immunodeficiency

Severe combined immunodeficiency (SCID) describes an immunological syndrome that arises from numerous genetic defects leading to the absence of humoral and cellular immunity in infants [118]. If left untreated, SCID

results in a fatal outcome from multiple and severe infections within the first few years of life. The incidence of SCID in the United States is approximately 1 in 58,000 infants [119].

The most prevalent type of SCID (approximately 45%–50%) is X-linked (X-SCID), in which there is a defect in the common gamma chain of the receptors for interleukin-2, -4, -7, -9, -15, and -21 that affects the development of T and NK cells [120]. It has an X-linked inheritance that results in phenotype expression predominantly in males. The second most prevalent SCID is genetic defects in the recombinase-activating genes [119]. These genes are responsible for the formation of T- and B-cell antigen receptors, which lead to nonfunctional T and B cells. NK cells are not affected. The next most prevalent SCID is the defect in adenosine deaminase (ADA)-SCID. ADA is an enzyme involved in purine metabolism [120]. ADA-SCID results in the accumulation of deoxyadenosine and deoxyadenosine triphosphate, which is toxic to lymphocytes [121]. As a frontline treatment, patients with ADA-SCID receive enzyme replacement therapy (ERT) using polyethylene glycol-conjugated adenosine deaminase (PEG-ADA). There are significant side effects including lymphoproliferative disorders, anemia, and pulmonary insufficiency [121] as well as a decreased thymus T-cell output, reduced T-cell function, and B-cell deficiencies over time [122–124].

Allogeneic hematopoietic stem cell transplantation is the best option for treatment for SCID. Bone marrow mobilized PBSCs from related, unrelated, or cord blood grafts are options for transplantation in patients with SCID. The first transplantation for SCID occurred in 1968 [125]. In 1968–2005 in Europe, patients who received a related genoidentical donor had a long-term survival of 90%, which was higher than for patients who had a related phenoidentical, mismatched related, or unrelated donor [126]. Patients who received a transplantation between 1995 and 2005 did better potentially as a result of improvement in transplantation care. Patients transplanted before age 6 months did better than did patients transplanted at age 1 year or older. In addition, outcomes were improved when patients did not have respiratory damage or viral infection before transplantation.

The Primary Immune Deficiency Treatment Consortium of 41 North American centers reported on 240 patients with SCID who received transplantations from 2000 to 2009 [127]. Grafts were either unmodified or T-cell depleted. Most patients transplanted with grafts from matched sibling donors or mismatched related donors did not receive conditioning; however, patients receiving grafts from unrelated donors or cord blood grafts received myeloablative conditioning, reduced intensity conditioning, or immunosuppression. The overall survival rate at 5 years was 74%. Patients receiving a matched sibling donor had a survival rate of 97%, whereas patients receiving an unrelated cord blood graft had the lowest survival rate of 58%. Similar to the finding from the European group, patients who were younger ($\leq$3.5 months) fared better. Patients older than 3.5 months at the time of transplant and with active infections did poorly.

Although allogeneic transplantation is the treatment of choice, the overall success depends on finding an appropriate donor. Gene therapy offers an alternative treatment in which autologous hematopoietic stem cells are used in association with gene therapy to correct the genetic defect. Gene therapy has been used for X-SCID and ADA-SCID. ADA-SCID was the first to be molecularly identified, and patients with ADA-SCID were the first to be treated with gene therapy in the early 1990s. No significant clinical improvement was seen; however, the trial demonstrated that gene therapy could be delivered safely. The patients with ADA-SCID continued to be treated with PEG-ADA, which may have accounted for the inability to determine the efficacy of the gene therapy. Subsequently, other trials using improved viral vectors and conditioning regimens were conducted to treat patients with ADA-SCID [128–131]. Approximately 40 patients with ADA-SCID have been treated, with significant improvement in immunity without ERT or allogeneic transplantation. These studies confirmed the safety of gene therapy for patients with ADA-SCID and demonstrate their significant clinical improvement.

Twenty patients with X-SCID were treated with autologous hematopoietic stem cells that were modified using gene therapy [132,133]. Significant clinical improvements were seen with T-cell reconstitution and improved B-cell function. However, 5 of the 20 patients developed leukemia [134,135]. The viral insertion site was the cause of the leukemia owing to insertional oncogenesis. This result led to the development of new retroviral and lentiviral vectors that appear to have no genotoxicity [136].

Hematopoietic Stem Cell Transplantation for Tolerance Induction

Immune tolerance is a state in which the immune system accepts donor tissues or organs but retains the ability to respond to foreign antigens such as bacteria or viruses. The induction of donor-specific tolerance is the ultimate goal for solid organ and composite tissue transplantation. This type of tolerance would eliminate the need of life-long immunosuppression with all of the associated side effects and prevent chronic rejection. Mixed chimerism is a state in which the hematopoietic and immune systems of an allogeneic hematopoietic stem cell transplant recipient

contain both donor and recipient cells. Mixed hematopoietic chimerism was first demonstrated to be associated with tolerance in fraternal twin cattle in 1945 [137]. Animal studies, including rodent, pig, dog and nonhuman primate, demonstrated hematopoietic mixed chimerism and tolerance induction for tissue and organ transplantation [138–142]. Because of these preclinical studies, several clinical trials for tolerance induction for kidney transplantation were performed [143–145]. Data from the three centers showed that donor hematopoietic stem cell infusion is feasible to induce tolerance for kidney organ graft. Durable to transient chimerism was established in some of the recipients with withdrawal of immunosuppressants. These results are promising; however, they represent only short-term success. Additional improvements in preconditioning to reduce toxicity and methods to maintain chimerism are needed. In addition, hematopoietic stem cell infusion to induce tolerance needs to be expanded beyond kidney transplants to other tissues and organs.

Hematopoietic Stem Cell Transplantation for Autoimmune Diseases

Autoimmune diseases are clinical conditions that result from the loss of self-tolerance. This includes the failure of both peripheral and central tolerance, which results in the generation of autoreactive T and B cells that damage tissues and organs. Approximately 5% of the population is affected by autoimmune diseases. Autoimmune symptoms can be managed but not cured by conventional treatment, which include antiinflammatory and immunosuppressive drugs. There is a group of autoimmune patients who express severe disease and are resistant to standard treatment. Morbidity and mortality are high for these patients; therefore, an alternative therapy is needed to improve their quality of life. Autologous hematopoietic stem cell transplantation has been evaluated for severe forms of autoimmune diseases. The most promising results have been seen for multiple sclerosis and systemic sclerosis [146]. There has been limited success for Crohn disease and type 1 diabetes [146]. However, significant adverse events, including relapse and mortality, were noted for autologous hematopoietic stem cell transplantations for systemic lupus erythematosus, rheumatoid arthritis, and juvenile idiopathic arthritis [147–149].

The rationale for autologous hematopoietic stem cell transplantation for autoimmune diseases is that it eliminates autoreactive immune cells by conditioning regimens and resets the immune system with the transplantation of hematopoietic stem cells.

Although autologous hematopoietic stem cell transplantation has been shown to reestablish self-tolerance, there are relapses that may be associated with the original genetic risk for autoimmune disease. Allogeneic hematopoietic stem cell transplantations establish a donor immune system in the recipient, potentially offering a more definitive cure. However, the risk for GVHD and the use of ablative conditioning treatments with their associated morbidity and mortality limit the widespread use of allogeneic stem cell transplants [150]. The current data suggest that the earlier that hematopoietic stem cell transplantation is performed, the more benefit is seen for the patient with autoimmune disease. A better understanding is needed of the mechanisms involved with hematopoietic stem cell transplantation in autoimmune diseases.

CONCLUSION

The hematopoietic stem cell is the most highly studied and probably best understood of all stem cell populations. Since the initial identification of the hematopoietic stem cell, its physical and functional properties have been extensively studied in multiple animal models. All of this knowledge has been translated into the clinic for use in treating bone marrow failure, malignancies, immune tolerance, and autoimmune diseases. Hematopoietic stem cells can be isolated from multiple sources including bone marrow, peripheral blood, and cord blood. This makes stem cells easily available in sufficient numbers for clinical use. These properties make hematopoietic stem cells a valuable therapy that can have widespread use in regenerative medicine.

List of Acronyms and Abbreviations

ADA Adenosine deaminase
ADA-SCID Adenosine deaminase-severe combined immunodeficiency
AGM Aorta-gonad-mesonephros
CFU-s Colony forming cell-spleen
CGL Chronic granulocytic leukemia
CML Chronic myeloid leukemia
DNA Deoxyribonucleic acid

GVHD Graft versus host disease
GVT Graft versus tumor
HLA Human leukocyte antigen
JIA Juvenile idiopathic arthritis
Lin− Lineage negative
LSK $lin^{-}/Sca\text{-}1^{+}/c\text{-}Kit^{+}$
MDR Multi drug resistance
NK Natural killer
PBSCs Peripheral blood stem cells
PEG-ADA Poly ethylene glycol conjugated adenosine deaminase
RA Rheumatoid arthritis
RAG Recombinase-activating genes
$Rho123^{lo}$ Rhodamine 123 low
SCID Severe combined immunodeficiency
SLAM Signaling lymphocyte activation molecule
SLE Systemic lupus erythematosus
SP Side population
X-SCID X linked severe combined immunodeficiency

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CHAPTER

14

Mesenchymal Stem Cells

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INTRODUCTORY OVERVIEW

In the development of stem cell–based therapeutic platforms for tissue regeneration, the selection of which type of stem cell to use will be enormously important. Adult mesenchymal stem cells (MSCs) are considered one of the most promising tools for cell and cell-based gene therapy in bone repair [1]. The best-known source of MSCs in adult humans is the bone marrow (BM) compartment. Other sources of MSCs have been identified, including adipose tissue, dermal tissue, intervertebral disc, various dental tissues, human placenta, cord blood, and peripheral blood, although the latter finding is still debated [2].

In cell-based therapies, the culture expansion stage is extremely costly and time consuming; in addition, in many cases cells may lose their multipotentiality in vivo and fail to meet the desired goal. It was reported that cultured human MSCs (hMSCs) can undergo spontaneous transformation as a consequence of in vitro expansion [3]. Moreover, culture expansion attenuated the homing ability of MSCs after systemic infusion in irradiated mice [4], which implies that MSCs may lose some of their natural stem cell characteristics after expansion in vitro. Other investigators proposed that all known characteristics of MSCs may be an outcome of the culture stage and do not truly represent the actual characteristics of MSCs residing in vivo at the BM niche [5].

The isolation of an hMSC-enriched population requires an efficient and reproducible method. We reported that we used the CD105-based immunoisolation method to obtain a fresh noncultured population of hMSCs, and we showed these cells' osteogenic potential both in vitro and in vivo [6]. In additional studies, a positive selection method was implemented as well: immunoisolation of MSCs with antibodies directed against the Stro-1 [7,8] and CD146 [9] markers.

One striking feature of MSC therapy is cumulative data on the tolerance shown by the host to allogeneic MSCs. The mechanisms by which this immunotolerance exist are complex and have not yet been thoroughly identified. It has been shown that there is a low expression of alloantigens by MSCs; this may involve pathways (dependent on or independent of cell contact) that are modulated by the secretion of soluble factors such as interleukin (IL)-2, IL-10, transforming growth factor-β1 (TGFβ1), prostaglandin E_2 (PGE2), hepatocyte growth factor (HGF), and others. Immune system cells such as dendritic cells (DCs) and T cells, have also been shown to be affected by the presence of MSCs in mixed lymphocyte cultures [10]. The inhibitory role of MSCs and the value of MSC cotransplantation in allogeneic hematopoietic stem cell (HSC) transplantation were thoroughly examined by Troeger et al. [11].

Several protocols have been established to enable the regeneration of large bone defects using hMSCs that have been expanded in culture. These cells differentiate into osteogenic cells; as vehicles, they deliver a therapeutic gene product such as one of the bone morphogenetic proteins (BMPs). This approach requires the genetic modification of MSCs to overexpress a transgene encoding for an osteogenic gene. BMP-2 has been widely used for this purpose [12–14], as have other members of the BMP family such as BMP-4, BMP-6, and BMP-9 [15–20]. In addition, MSCs have been implemented in regeneration of the heart (cardiac muscle and vascular system), skeletal muscle, nerve, liver, and pancreas; regeneration of cardiac tissue is foremost [21–29].

Resembling the MSCs, exosomes secreted from those cells support tissue homeostasis and correct cell functioning and regeneration, perhaps providing validation for the therapeutic ability of MSCs in a broad variety of diseases. A

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growing amount of MSC research has been dedicated to deciphering the MSC secretome, if the soluble factors or the factors released in extracellular vesicles (EVs), such as exosomes and microvesicles (MVs) are the active factors [30].

Although the embryonic origin of MSCs remains unclear, multiple methods have been developed to differentiate MSCs derived from induced pluripotent stem cells (iMSCs). These include treatment with specific factors [31] using cell isolation based on specific surface markers [32] or biomaterials-directed differentiation [33,34]. iMSCs were shown to be functional and expandable; however, to advance the application of these cells in clinic, major studies have to be performed to make the differentiation process more efficient and address safety concerns. Although there are several indications that iMSCs are actually less tumorigenic than the reference standard, BM-derived mesenchymal stem cells (BM-MSCs) [31,35], iMSCs must be further explored.

DEFINITION OF MESENCHYMAL STEM CELLS

BM was the first tissue identified to be a source of plastic-adherent fibroblast-like cells that develop colony-forming unit—fibroblasts (CFU-Fs) when seeded into tissue culture plates [36]. These cells, which were originally designated stromal cells, elicited much attention and were the focus of thousands of studies aimed at finding a pure cell population that could eventually be used for regenerative purposes. In these studies, cells were isolated using a variety of methods (discussed later in this chapter) and given names such as MSCs, mesenchymal progenitors, and stromal stem cells. A committee of the International Society for Cytotherapy suggested the name "multipotent mesenchymal stromal cells" [37]; however, most scientists simply refer to these cells as "MSCs."

The precise characterization of MSCs remains a matter of debate. Nevertheless, MSCs are widely defined as a plastic-adherent cell population that can be directed to differentiate in vitro into cells of osteogenic, chondrogenic, adipogenic, myogenic, and other lineages [5,38,39]. As part of their stem cell nature, MSCs proliferate and develop into daughter cells with the same phenotype and pattern of gene expression, thereby maintaining the "stemness" of the original cells. Self-renewal and differentiation potential are two criteria that define MSCs as real stem cells; however, these characteristics have mainly been demonstrated, both in bulk and at the single-cell level, after in vitro manipulation, and there is no clear description of the characteristics displayed by nonmanipulated MSCs in vivo [40]. So far, only one study has claimed to demonstrate the in vivo self-renewal property of MSCs identified by melanoma cell adhesion molecule (MCAM)/CD146 surface markers [41].

In contrast to other stem cells such as HSCs, which are identified by the expression of the CD34 surface marker, MSCs appear to lack one unique marker. The CD105 surface antigen (endoglin) has been used to isolate hMSCs from BM, enabling the characterization of freshly isolated hMSCs before culture. Distinct expression profiles of certain surface antigens, such as CD45 and CD31, have also been demonstrated in freshly isolated hMSCs, whereas expression of these molecules is lower in culture-expanded hMSCs [6]. From these data, one can infer alterations that hMSCs may undergo during the culture process [42]. Findings of other studies, however, indicated that CD146 or STRO1 could serve as unique markers for MSCs [7,8].

In several studies, cultured MSCs have been characterized either by using cell surface antigens or by examining the cells' differentiation potential. In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria by which one can define human multipotent mesenchymal stromal cells (also known by the abbreviation "MSCs"): (1) these multipotent mesenchymal stromal cells must be plastic-adherent when maintained in standard culture conditions and form CFU-Fs; (2) these cells must express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14, CD11b, CD79α, or CD19, and human leukocyte antigen—antigen D-related surface molecules; and (3) these cells must differentiate into osteoblasts, adipocytes, and chondroblasts in vitro [37]. The term "multipotent mesenchymal stromal cells" refers to cultured cells, whereas the term "mesenchymal stem cells" is designated for stem cells yet to be identified in vivo.

THE STEM CELL NATURE OF MESENCHYMAL STEM CELLS

Stem cells are defined by their ability to self-renew and by their potential to undergo differentiation into functional cells under certain conditions. There is minimal evidence showing that such features exist in uncultured MSCs. The consensus is that cultured MSCs can self-renew and differentiate into osteoblasts, adipocytes, and chondroblasts in vitro. Whether these cells can also differentiate into tenogenic, myogenic, and numerous other cells types, as has been claimed in many studies, remains controversial. The debate on this subject originated from

evidence obtained during research into embryonic development. It is known that there is no common progenitor for bone and muscle tissues after sclerotome-myotome specification in somites [43], and hence it is unlikely that such a cell exists in the adult. Supporters of this view claim that MSCs reside in BM and can only develop into bone, cartilage, and adipose cells.

Other researchers claim that MSCs can be isolated from BM as well as other adult tissues and can be differentiated into epithelial, endothelial, hepatic, and neural cells [44–47]. However, studies have shown that MSCs isolated by plastic adherence from various tissues differ considerably in morphology, proliferative capacity, differentiation capability, and the ability to form bone in vivo [48]. Therefore, MSCs from different tissue sources may not be the same cell or they may contain a heterogeneous population of progenitors that can explain the variability in their characteristics. Efforts to find a "pure" or primitive multipotential stem cell across tissues has not been well-supported [49].

Findings of some studies suggest that the multipotentiality of MSCs can be demonstrated by direct injection into extraskeletal tissues such as the brain. It was claimed that an injection of hMSCs into the brain tissue of rats resulted in the cells' long-term engraftment and subsequent migration along pathways similar to those used by neural stem cells [50]. Other studies, however, showed that injected MSCs were rejected or fused to existing differentiated cells within the tissue [51,52]. The results of these studies further strengthened the illusiveness of finding a multipotential stem cell that may reside in all or most tissues. Nevertheless, this does not hinder the therapeutic potential of MSCs from BM or other tissues. It may well be that MSCs, as they are currently used, are a heterogeneous population of cells including both multipotent and progenitor cells with unique capabilities of tissue regeneration and immune response modulation. These capabilities could be exerted via paracrine effects [53], autocrine involvement in tissue repair [54], or both.

WHICH TISSUES CONTAIN MESENCHYMAL STEM CELLS?

The embryonic origin of MSCs is unclear; however, some findings indicate a possible origin of MSCs in a supporting layer of the dorsal aorta in the aorta-gonad–mesonephros region [55]. Consistent with these findings, MSCs or MSC-like cells were found circulating within human fetal blood [56].

MSCs that comprise the stroma-supportive system of BM along with endothelial cells and adipocytes are the most well-studied [57]. An MSC population has also been identified in the BM of the craniofacial complex [58]. In adults, MSCs appear to be "resident" stem cells in many tissues, and they function in the normal turnover of these tissues. Numerous studies have demonstrated the presence of MSCs or MSC-like cells within other tissues such as adipose tissue (adipose tissue–derived stem cells [ASCs]) [59], dermal tissue [60], the intervertebral disc [61], amniotic fluid [62], various dental tissues [63], human placenta [64], cord blood [65], the temporomandibular disc [66], and peripheral blood [67].

ASCs are similar to BM-derived MSCs both morphologically and immunophenotypically; however, ASCs form more CFU-Fs when plated in culture [68]. Adipose tissue is an attractive source of MSCs for regenerative medical purposes; it is relatively easy to obtain, can be collected with the use of local anesthesia, and is associated with minimal discomfort and risks [69].

There has been considerable interest in investigating the hypothesis that MSCs are in fact pericytes that abut the basement membrane and engulf endothelial cells in the microvasculature. Several studies have supported this hypothesis [70], yet there is no agreement among scientists as to whether all BM pericytes are MSCs or whether pericytes found in other tissues are also MSCs. Caplan speculated that all MSCs are pericytes [71], and indeed da Silva Meirelles et al. showed that pericytes from adipose tissue are similar to adipose-derived MSCs in gene expression profiles [72]. However, Blocki et al. showed that only a subset of BM pericytes can be considered MSCs [73]. Sacchetti et al. were the first to determine that $CD146^{+}$ perivascular cells in BM are MSCs [41]. They compared MCAM/CD146-expressing cells from different human tissues and analyzed their differentiation potentials and transcriptomes [74]. The results indicated that although the various cells were associated with microvessels, i.e., pericytes, they differed significantly in their skeletogenic differentiation pattern and their transcriptomic profile. Moreover, Guimarães-Camboa et al. analyzed pericytes from multiple tissues in a mouse model and found that these cells (identified by T-Box 18 expression) did not behave like stem cells in vivo and did not differentiate to other cell types. However, these cells did behave like MSCs in vitro [75].

Based on current knowledge, it is safe to state that MSCs are associated with the microvasculature and that different tissues contain MSC-like cells that have some characteristics in common and others that differ.

MESENCHYMAL STEM CELL ISOLATION TECHNIQUES

Application of MSCs requires isolation of these cells and directing cell differentiation into the appropriate lineage. Since the 1980s [36], Ficoll−Paque density-gradient media have been used to separate mononuclear cells (MNCs) from red blood cells in BM. The MNCs are then collected and seeded in medium containing 10% fetal bovine serum (FBS) at a density of $10-15 \times 10^5$ cells/cm^2 growth area [38]. Adherent spindle-shaped cells appear within 48 h after the initial seeding, and the estimated percentage of MNCs ranges from 0.001% to 0.01%.

Since then, many approaches have been proposed to isolate MSCs. Ficoll Paque−based isolation may be used to isolate MSCs from BM as well as from peripheral blood; this approach has been used in most clinical trials performed to date [76]. Enzymatic treatment of adipose tissue with collagenase is performed to produce ASCs shown to have the high potential for in vitro expansion and in vivo differentiation [59,77,78]. A method based on the biophysical properties of MSCs in suspension under fluidic conditions has been proposed as well [79].

Major disadvantages of these methods are the impurity of the resulting cell population and the need to culture cells before application. The solution to these downsides will include isolation of cells based on the intrinsic properties of MSCs, avoiding culturing, and the generation of immortalized cell lines. Immunoisolation is a method to isolate noncultured MSCs based on cell surface markers. Several studies have employed the "positive selection" technique of immunoisolating MSCs by using antibodies directed against endoglin (CD105) [6], Stro-1 [7,8], CD146 [9], and other MSC markers. Immunodepletion, the "negative selection" of unneeded cell populations, including residual hematopoietic cells, may be used to enrich the MSC population further, especially in combination of the based on different surface markers [80]. Busser et al. compared the use of different cell markers to immunoisolate MSCs from BM and adipose tissue; they found that different antibodies yielded MSC populations that differed in immunophenotype and in differentiation and immunoregulatory capabilities [81].

A major challenge is the development of the large-scale production of good clinical practice−quality MSCs. Clinical-grade cells have to be safe for use; hence, there is a need for microbe-free closed-circuit systems [82]. An additional concern would be the use of xenogenic reagents such as FBS. Several human reagents have been used, including fibroblast growth factor [83] and platelet lysate, which has been shown to produce MSCs with various immunocapabilities [84]. Fekete et al. proposed five large-scale, two-step MSC isolation protocols, which yield as much as 6×10^9 MSCs per BM aspirate [85].

MESENCHYMAL STEM CELL EXOSOMES

In several cases, a positive regenerative and healing effect was evident after systemic or local treatment with MSCs. Verifying the actual presence of these cells at the site of injury, however, is sometimes difficult or insurmountable. In fact, a correlation between administration of cells and observed improvement cannot always be recognized. Based on this difficulty of obtaining proof of the cells' presence, it has been speculated that it is not MSCs that differentiate into tissue-specific mature cells, but instead secreted factors, more precisely exosomes. Resembling their cell source, the MSC, these secreted exosomes support tissue homeostasis and correct the courses of cell function and regeneration, as required. These activities may validate the therapeutic ability of MSCs in a broad variety of diseases. Increasing numbers of MSC research studies have focused on deciphering the MSC secretome, the soluble factors, or the factors released in EVs, such as exosomes and MVs.

EVs consist of subgroups (all secreted membrane-enclosed vesicles) that include exosomes, ectosomes, MVs, microparticles, apoptotic bodies, and other EV subsets. In a position statement, the International Society for Extracellular Vesicles [86] declared that there are no specific markers of EVs with which we can establish basic standards for identification; the organization speculated that this is because EV secretion probably varies greatly depending on the environment. Thus far, there is no solid evidence to affirm that different classes of EVs represent distinct biological entities. Morphology and size guide us to discriminate among various exosomes; specifically, nanospheres are composed of a lipid bilayer similar to a liposome (40−100 nm), MVs or ectosomes are derived from the shedding of the plasma membrane (100−1000 nm), and apoptotic bodies (1−5 μm) are released from the cell membrane as blebs during apoptosis.

Similar to exosomes in general, MSC exosomes transport a composite load that contains nucleic acids, proteins, and lipids. Unique gene products number 857 and more than 150 microRNAs (miRNAs) have been identified, although whether this diverse content is carried by one exclusive type of exosome or is split within different types of exosomes has yet to be determined [87].

The exosomal proteins, RNAs, and lipids mentioned in several published and unpublished studies have been cataloged in ExoCarta, a database that aims to identify specific molecular signatures of tissue/cell type−derived exosomes [88]. ExoCarta contains a list of 100 gene-encoding proteins most often identified in exosomes. The top five protein-encoding genes (index >90) include the following:

1. CD9, which encodes a protein (a member of the tetraspanins) that functions in many cellular processes including differentiation, adhesion, and signal transduction. Expression of the gene has a critical role in suppressing cancer cell motility and metastasis [RefSeq, Jan 2011].
2. HSPA8, which encodes a constitutively expressed member of the heat shock protein 70 family. This protein functions as a chaperone and binds to nascent polypeptides to facilitate correct folding. It also functions as an adenosine triphosphatase in the disassembly of clathrin-coated vesicles during transport of membrane components through the cell [RefSeq, Aug 2011].
3. PDCD6IP (or ALIX), which encodes a protein that functions within cytosolic protein complexes and endosomal sorting complexes required for transport in the abscission stage of cytokinesis, in endosomal vesicle formation, and also in enveloped virus budding. PDCD6IP is also involved in the membrane-shaping phase of endocytosis, binding to endophilins [RefSeq, Jan 2012].
4. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) encodes a protein of the GADPH family, which has been acknowledged to be a moonlighting protein based on its ability to perform multiple functions. The GAPDH product catalyzes a critical step in carbohydrate metabolism: the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide. In addition, in the cell nucleus, the encoded protein has been identified to have uracil DNA glycosylase activity [RefSeq, Nov 2014].
5. Actin β is a major constituent of the contractile apparatus and one of the two nonmuscle cytoskeletal actins. This gene encodes one of six different highly conserved actin proteins involved in cell motility, structure, and integrity [RefSeq, Jul 2008].

Having a number of common protein markers for exosome identification is a step forward in the future implementation of exosomes for therapeutic applications, even though the isolation method is not yet well-confirmed. Established procedures for separating exosomes include ultracentrifugation, density gradient separation, chromatography, immune-affinity capture (IAC), and polymer-based precipitation. With the use of all of these techniques, it is possible to isolate vesicles according to their diameters and markers. IAC appears to be the most effective process based on the number of exosome-related proteins identified on the purified fraction. Nevertheless, there may be situations in which there are no suitable antibodies to determine exosomal markers; in such cases, the use of density-based separation will be advantageous for exosome isolation [89].

The notion that growth factors, chemokines, and cytokines would be the molecules responsible for the systemic and local effects of MSCs, such as immunomodulation, was discarded after several published reports showed that the absence of those molecules did not affect the immunomodulatory activity of MSCs. EVs have been reported since 2010 to be effective therapeutic mediators in MSCs, when Lai et al. revealed that MSCs secrete a specific class of EVs known as exosomes, with diameters ranging from 40 to 100 nm and bearing exosome-associated proteins such as ALG-2-interacting protein X, tumor susceptibility gene 101, and the tetraspanin proteins. After succeeding in deriving and characterizing fetal hMSCs, this group also showed that these cells' secretion was cardioprotective in a mouse model of myocardial ischemia/reperfusion injury. Analysis of the secretion by high-performance liquid chromatography revealed the presence of microparticles with radii ranging from approximately 50 to 65 nm. This population of microparticles was cardioprotective at about one-tenth the dosage of crude secretion [90].

The exosomes contain, carry, and dispense a range of small molecules released from stem cells to nearby cells, thus enabling the uptake of enzymes and nucleic acids through the endocytosis of circulating exosomes, especially by cells in injured tissue, which often has a lower pH than normal tissue; this is a favorable condition for exosome uptake. MSC exosomes not only interact with nearby cells; via blood and other biological fluids, these exosomes can migrate to distant targets. The recipient cell response may differ according to what molecules enclosed in the exosomes reached the objective [91].

Evidence continues to grow showing that MSC-derived EVs produce beneficial effects in several disease models, such as heart conditions and kidney, brain, and skeletal injuries, and in areas of intervention, including aspects of regenerative medicine.

Exosomes released from MSCs and enriched with miRNAs have a definitive role in the heart and blood vessels, exerting antiapoptosis and antiinflammatory effects, cardiac regeneration, and neovascularization, which are considered components of whole molecular mechanisms lying behind the therapeutic potential of MSC

transplantation, according to a thorough review by Huang et al. [92]. Results of preclinical studies suggested that exosomes can be used to treat cardiovascular diseases (CVDs) such as acute myocardial infarction (AMI) and stroke when derived from human, mouse, or rat MSCs. Different experimental models have been used, and cardiac progenitor cell (CPC)-derived exosomes, embryonic stem cell (ESC)-derived exosomes, and $CD34^+$ stem cell—derived exosomes significantly reduced infarct size, restored cardiac function, and stimulated angiogenesis in ischemic zones. CPCs significantly suppressed apoptosis, stimulated angiogenesis, and improved cardiac function in a rat model of AMI. In vitro experiments demonstrated that MSC-derived exosomes augmented the survival and proliferation of CPCs obtained from the infarcted area. Altogether, these studies provide compelling evidence that exosomes derived from a variety of stem cells exert beneficial effects on animal models of CVD [93].

Growth factors known for their proangiogenic activity, such as vascular endothelial growth factor (VEGF), TGFβ1, and IL-8, were identified in exosomes derived from MSCs by proteomic analysis. In addition, it was reported that MSC-derived EVs are also rich in HGF, a transcription factor that stimulates the proliferation and migration of endothelial and vascular smooth muscle cells involved in proangiogenic pathways. Human T-cell factor 4 (TCF4) is a key downstream effector of Wnt signaling, a canonical pathway that has a central role in vascular development and in determining and maintaining the phenotype and functional properties of human stem cells. Therefore, intercellular transmission of EVs containing VEGF, TGFβ1, IL-8, HGF, and TCF4 may have both proangiogenic and prosurvival effects [94].

A metaanalysis performed by Zhang et al. confirmed the clinical effectiveness of using exosomes in ischemia/reperfusion injury, based on reports published between January, 2000 and September, 2015, and indexed in the PubMed and Web of Science databases. The authors' analysis of the data they gathered validated the therapeutic potential of MSC-secreted exosomes in improving heart function. Still, more steps are required to understand these exosomes' mechanism of action fully and ensure their therapeutic safety; in addition, clinical trials are required to optimize this cardiac regeneration approach after ischemic events [95].

Mesenchymal stromal cells promote recovery after acute kidney injury (AKI), and phenotypic changes have been associated with the EVs implicated in the process. Knowing that miRNAs are potential candidates for cell reprogramming toward a regenerative phenotype, Collino et al. conducted a study to evaluate whether miRNA deregulation inhibits the regenerative potential of mesenchymal stromal cells and derived EVs in a model of glycerol-induced AKI in severe combined immunodeficient mice. The results showed that miRNA depletion in mesenchymal stromal cells and in EVs significantly reduced their intrinsic regenerative potential in AKI [96]. Furthermore, when AKI occurs, dedifferentiation of the tubular cells initiates cell regeneration, involving HGF by modulating cell dedifferentiation. MSC—derived MVs deliver RNA into injured tubular cells and alter their gene expression, thus regenerating these cells. It was reported that in damaged tubular cells, HGF synthesis induced by MVs via RNA transfer facilitates cell dedifferentiation and growth, which are important regenerative mechanisms [97].

A study of the growth-promoting effect of EVs derived from different MSC sources on neurons was undertaken by the Khoury group. The researchers compared the effect of human menstrual MSCs (MenSCs), which were mediated via cell-to-cell contact by their total secretomes or by secretome-derived EVs, on neuritic outgrowth in primary neuronal cultures. The researchers showed that MSCs purified from menstrual fluid released MVs and exosomes, and that exosomes present in conditioned medium (CM) exerted a beneficial effect on neuron outgrowth, an action that could not be fully reproduced by exosomes derived from other MSC sources, such as BM-, chorion-, and umbilical cord—derived MSCs, or by other MenSC culture components [98].

Having demonstrated the neuroprotective potential of systemically administered MSCs in a preclinical animal model of hypoxic-ischemic brain injury in ovine fetuses, another research group investigated whether these protective effects can be exerted by MSC-derived extracellular vesicles (MSC-EVs) in a preclinical model of preterm hypoxic-ischemic brain injury. Ovine fetuses were subjected to global hypoxia-ischemia, followed by in utero intravenous administration of MSC-EVs. The systemic administration of MSC-EVs improved brain function by reducing the total number and duration of seizures and by preserving baroreceptor reflex sensitivity. These protective effects were accompanied by a tendency to prevent hypomyelination [99]. These results are relevant because they show that a cell-free preparation composed of neuroprotective MSC-EVs could eventually become a substitute for MSCs, avoiding the potential risks involved in the systemic administration of living cells.

With respect to bone regeneration, Nakamura et al. [100] suggested that MSC-derived exosomes can promote skeletal muscle regeneration by enhancing myogenesis and angiogenesis; these researchers considered exosome-enclosed miR-494 to be an important mediator of this response. In addition, Narayanan et al. [101] demonstrated the potential of exosomes released by MSCs cultured under osteogenic conditions: In both two- and three-dimensional (type I collagen hydrogels) cultures, these exosomes induced lineage-specific differentiation of undifferentiated hMSCs both in vitro and in vivo. Results of the study also showed that exosomes can bind to extracellular

matrix (ECM) proteins such as type I collagen and fibronectin [102]. Work published by Furuta et al. focused on exosomes as a valuable addition to cell-to-cell communication. This study evaluated the role of exosomes isolated from MSC-CM in the fracture healing process of $CD9^{-/-}$ mice, a strain known to produce reduced levels of exosomes. Injection of MSC-EVs accelerated fracture healing in $CD9^{-/-}$ mice, which would otherwise be naturally delayed, whereas injection of exosome-free CM had no rescue effect [103].

Human embryonic MSC-derived exosomes were tested in an animal model of cartilage repair. Osteochondral defects were created on both distal femurs in adult rats, and treatment consisted of intraarticular injections of exosomes or phosphate-buffered saline (PBS), administered after surgery and thereafter weekly for 12 weeks. Overall, the exosome-treated defects displayed an enhanced gross appearance and improved histological scores compared with the contralateral PBS-treated defects. In the exosome-treated defects, cartilage and subchondral bone exhibited complete restoration and complete bonding to adjacent cartilage, as well as an ECM deposition that closely resembled that of an age-matched healthy control [104].

Designating a major role to exosomes in mediating the therapeutic and paracrine effects of MSCs offers additional advantages. There is less immunogenicity than that found with parental cells bearing a low content of membrane-bound proteins, and exosomes can be stored at −20°C for 6 months with no loss of biochemical activity. The cytokines, growth factors, transcription factors, and RNAs contained inside the exosomes are protected from degradation in vivo, thereby potentially preventing some of the problems associated with small soluble molecules, which degrade rapidly [105].

IMMUNOMODULATORY EFFECTS OF MESENCHYMAL STEM CELLS

Several studies have shown that MSCs escape recognition by the immune system and inhibit immune responses [106]. Modulation of the immune system has been detected in both BM-MSCs and ASCs. This property of MSCs facilitates the clinical use of MSCs in an allogeneic manner in both immune disease and regenerative medicine approaches. Numerous clinical trials involving the use of MSCs have been initiated: 38 to study the effect of MSCs on graft-versus-host disease (GVHD), 22 to treat multiple sclerosis, 14 to treat different forms of anemia, 21 to achieve bone regeneration, and 47 to treat patients with cirrhosis or facilitate liver transplantation [107].

Various mechanisms have been suggested to explicate how MSCs prevent allogeneic rejection among different species [108]: weak immunogenicity; interference in the maturation and function of DCs [10]; abolishment of T-cell proliferation by various pathways including Fas/Fas ligand−induced T-cell apoptosis [109]; and interaction with natural killer (NK) cells in cell-to-cell contact or by releasing soluble secreted factors [110]. Although there have been discrepancies, probably owing to differences in implementing experimental systems, most reports have indicated no or low expression of major histocompatibility complex class II proteins [111]. At the molecular level, many researchers believe that MSCs migrate to sites of injury where they secrete various modulator molecules including immunoregulatory and trophic signals [53]. Najar et al. [112] proposed a description of a regulatory network in which each immune mediator affects and is affected by others; accepted major modulators include HGF, TGF-β [113], nitric oxide [114], PGE2 [115], and IL-10 (the central player in the network). IL-10 is not secreted by MSCs, but by lymphocytes [116] in response to MSC secretion of HGF, PGE2, and other factors [81]. IL-10 has a pleotropic effect that includes diversion of the type 1 T helper/type 2 T helper balance and induction of regulatory T-cell differentiation while regulating antigen-presenting cell activity [117]. iMSCs retain some of the characteristic MSC immunomodulatory capabilities [118], including NK cell downregulation [119].

Exosomal signaling has been shown to participate in MSC immunomodulation [120,121]. Blazquez et al. reported that exosomes secreted by ASCs are able to inhibit the proliferation of stimulated T cells harvested from peripheral blood [120]. The contribution of miRNA molecules has been documented as well: for instance, in an MSC-modulated hepatitis C model [122] and an AKI model [96].

The timing of MSC therapy favoring immunomodulation is highly important. Wang et al. [123] described the plasticity of MSCs in response to the three phases of inflammation: inflammatory, reparative, and remodeling. Because the response of MSCs is affected by the stimulation of immune cells, there is an optimal window during which therapy should be applied. For instance, MSC infusion will not affect GVHD if it is performed during BM transfusion, before inflammation has developed [124], although MSCs can modulate GVHD if administrated 2 days later [125]. We observed a similar effect when we used systemic MSC administration to model vertebral compression fracture (VCF) repair; repair worked only when the MSCs were administered 3 days after the creation of the VCF [54]. Ankrum et al. [126] suggested strategies to prolong the persistence of allogeneic MSCs, including low-dose immunosuppression, polymer capsulation, and even genetic modification of exogenously administrated MSCs.

Overall, the way in which MSCs avoid detection by the immune system has not been thoroughly elucidated. Additional soluble factors or cells may be found to exert a significant impact, and novel mechanisms may be revealed.

INDUCED PLURIPOTENT STEM CELL–DERIVED MESENCHYMAL STEM CELLS

The prevalence of musculoskeletal disorders in older persons, taken together with challenges associated with the aging of the world population, has motivated researchers to investigate the impact of aging on the regenerative properties of MSCs, because of these cells' potential as an autologous treatment [127]. Although conflicting results have been published, in a variety of organs the regenerative potential of MSCs appears to decline with advancing age [128]. Human ESCs, the first pluripotent stem cells to be discovered, were found to be capable of inducing mesenchymal tissue formation in vivo [129]. Although ESCs are believed to be immunoprivileged, they have been shown to possess immunogenicity after differentiation [130].

Many ethical questions and debates have arisen from the use of ESCs [131]. As a consequence, there is need for an alternative inexhaustible source of MSCs to treat various skeletal disorders. It has been awhile since induced pluripotent stem cells (iPSCs) were discovered [132], and they provide a feasible solution. The emergence of iPSC technology was saluted with enthusiasm because it provides a reliable technological tool for generating pluripotent cells from practically any somatic cell and, theoretically, for obtaining any differentiated cell type. Use of iPSCs provides relief from a heated ethics controversy over the use of the only other pluripotent stem cells available: ESCs. Also, the notion that less than a handful of genes could reprogram an adult cell into pluripotency was astonishing to the stem cell research community. This discovery provides an opportunity to generate and develop either autologous MSCs or an allogeneic off-the-shelf MSC product. The embryonic origin of MSCs remains unclear, although some studies indicate a possible origin for MSCs in a supporting layer of the dorsal aorta in the aorta-gonad-mesonephros region [55]. Consistent with these findings, MSC-like cells have been found to circulate in fetal human blood [56]. By overcoming the immunological and ethical problems associated with ESCs, use of iPSCs opens a new avenue for cell transplantation–based regenerative medicine [133]. Thus, iPSCs, which are aged cells that possess the properties of young/embryonic cells, could solve one of the bottlenecks for clinical cell therapy, which is the shortage of functional autologous MSCs [134].

Since 2005, several methods have been developed to differentiate pluripotent stem cells (including both ESCs and iPSCs) into MSCs with various efficiencies of differentiation [129]. Initially these methods were developed for ESCs and included OP9 coculture [135], embryoid body formation [129], and direct plating of human ESCs in MSC media [136] with cell sorting [137], scraping [138], or manual colony picking and expansion of spontaneously differentiated human ESCs based on morphology [139]. Some of these methods were applied to iPSCs, as well; specifically, embryoid body formation was used as a first step toward MSC differentiation [31,140]. In other studies, Dulbecco's Modified Eagle Medium or a similar medium was used to culture iPSCs for prolonged periods to initiate spontaneous differentiation toward the mesenchymal lineage [119]. New methods were also developed to induce well-controlled and efficient reprogramming of iPSCs to iMSCs, including treatment with specific factors [31], using cell isolation based on specific surface markers [32], or biomaterials-directed differentiation [34]. Unfortunately, there is no consensus method to differentiate iMSCs, and differences in protocols, however small, can result in variable differentiation potentials, expandability, and properties of the end point tissue that is formed using those cells [31]. Therefore, these is a need to unify the differentiation methods and elaborate multiple tests to evaluate each batch of differentiated cells, to develop these cells into a potential therapeutic agent and move them forward to clinical applications.

Shortly after their discovery, the value of iPSCs as tools for modeling diseases became widely appreciated in the general stem cell scientific community. One of the main advantages of iPSCs lies in the ability to derive these cells from the individual patient, expand the cells, differentiate them into the desired cell type, and recreate the patient's disease in a dish. This method is mostly relevant in cases of genetic diseases, in which a mutation is transferred from the patient's somatic cells to the iPSCs and then to the differentiated cells, allowing researchers to investigate the phenotype of target cells without the need to procure target tissue or cells from the patient. There are no known diseases that specifically originate in mutated or compromised MSCs. Multiple disorders of mesenchymal tissues, such as Marfan syndrome [141] and fibrodysplasia ossificans progressiva [142], have been studied using iPSC-mediated disease modeling; however, most of these models used direct reprogramming of iPSCs to the cell of interest without passing through the intermediate MSC stage [143].

Levi and colleagues showed that iPSCs can be directed toward osteogenic differentiation in the presence of BMP-2 protein [144]. In that study, no teratoma formation was observed, even though no differentiation of iPSCs was induced before implantation. Still, the question of the tumorigenicity of pluripotent stem cells remains unanswered [145], and it is less likely that iPSCs will be used in the clinical setting unless a well-controlled differentiation procedure is developed that rules out any chance of tumor formation. Nevertheless, when tumorigenicity was specifically studied and iMSCs were compared with BM-MSCs, iMSCs were found to be less tumorigenic than BM-MSCs [31]. These iMSCs homed to tumors with efficiencies similar to BM-MSCs but were much less prone than BM-MSCs to promote an epithelial–mesenchymal transition, invasion, stemness, and growth of cancer cells [35]. These observations might be explained by the much lower expression of receptors for IL-1 and TGFβ, downstream protumor factors, and hyaluronan and its cofactor TSG6, all of which contribute to the protumor effects of BM-MSCs [35]. All of these findings suggest that once standardized, iMSCs will have the potential to become a safer alternative to the traditional and well-established BM-MSCs for therapeutic applications, especially in patients with cancer.

List of Acronyms and Abbreviations

AKI Acute kidney injury
AMI Acute myocardial infarction
ASC Adipose tissue–derived stem cell
ATPase Adenosine triphosphatase
BM Bone marrow
BM-MSC Bone marrow-derived mesenchymal stem cell
BMP Bone morphogenetic protein
CFU-F Colony-forming unit–fibroblast
CPC Cardiac progenitor cell
CVD Cardiovascular disease
DMEM Dulbecco's modified eagle medium
ECM Extracellular matrix
ESC Embryonic stem cell
ESCRT Endosomal sorting complexes required for transport
EV Extracellular vesicle
GCP Good clinical practice
GVHD Graft-versus-host disease
HGF Hepatocyte growth factor
hMSC Human mesenchymal stem cell
IAC Immune-affinity capture
IL Interleukin
iMSC Induced-pluripotent stem cell–derived MSCs
iPSC Induced pluripotent stem cell
MenSC Menstrual mesenchymal stem cell
MHC Major histocompatibility complex
miRNA microRNA
MLC Mixed lymphocyte culture
MNC Mononuclear cell
MSC Mesenchymal stem cell
MSC-EV MSC-derived extracellular vesicle
MV Microvesicle
NAD Nicotinamide adenine dinucleotide
NK Natural killer
PBS Phosphate buffered saline
PGE2 Prostaglandin E_2
TCF4 T-cell factor 4
$TGF\beta_1$ Transforming growth factor-beta1
VCF Vertebral compression fracture
VEGF Vascular endothelial growth factor

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CHAPTER

15

Mesenchymal Stem Cells in Regenerative Medicine

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INTRODUCTION AND HISTORY

The body continuously changes, and this is controlled primarily by genetic factors. The same genomic program that brings the fertilized egg through a series of multiplication and differentiation changes to bring about the birth of a complete multitissued organism controls the continuous changes through neonatal, juvenile, and teen stages and all of adulthood. Individuals aged 10, 30, 50, 70, and 90 years exemplify this continuous genetic and distinctive process of change. Importantly, the later stage of this process, referred to as aging, is not a disease state but rather part of this genomically controlled continuum. The central feature of change is that tissue-specific progenitor cells divide and their progeny differentiate in a sequence of site-specific changes to both expand the dimensions of tissues and *replace* cells that naturally expire. Every cell in the body has a life span measured in minutes, weeks, or in some rare cases, years. With only a few exceptions, end-stage differentiated cells die within a fixed time frame. The progenitor cells for that expired cell must replace the expired cells; the rate of replacement controls whether the tissue will increase in size, be maintained, or experience atrophy as seen in old age.

Within this view, I proposed the scheme pictured in Fig. 15.1, recognizing that bone marrow contained osteochondral progenitors, which had been used for decades to repair bones [1–3]. Fig. 15.1 was established in schematic form to mimic what was known about the lineages of hematopoiesis [4], except that in 1988 we knew the most about the lineage progression on the left of Fig. 15.1 [5] and nothing about the lineages on the right. In addition, in reviewing prior experiments from Dr. A. Friedenstein's lab in Moscow and her own findings, Professor Maureen Owen also proposed the existence of osteochondral progenitors in a logic mirroring hematopoiesis [6]. The dogma of that era (the 1980s and early 1990s) was that there was only one progenitor or stem cell, the hematopoietic stem cell (HSC), in adult organisms. The full scope of Fig. 15.1 was clearly not envisioned in 1988 and the term mesenchymal stem cell (MSC) was considered by some to be provocative at best and by others to be outlandish. Because the focus of Fig. 15.1 was on the differentiation lineages, all of the research of that era focused on tissue engineering strategies to rebuild damaged tissues; most experiments and transplantations were formulated in the orthopedic sector with bone [7,8], cartilage [9,10], muscle [11,12], and tendon [13] being prominent.

Because the MSCs were isolated from marrow, it was hypothesized that these cells were the progenitors for the bone marrow stroma (the highly differentiated tissue that supports all of hematopoiesis) [14]. Although most preclinical models using MSCs were in the orthopedic sector (bone, cartilage, and tendon) in the early 1990s, the first clinical trial using human MSCs (hMSCs) conducted by my colleagues in hematology-oncology was to supplement bone marrow transplantations with culture-expanded hMSCs for patients with cancer and eventually for patients with gene defects in mesenchymal-linked processes or tissues [15–17]. The logic of that era was that the MSCs, like the HSCs, would "home" back to the marrow when infused into the bloodstream; once home, the MSCs would rebuild or enhance the marrow scaffold to accelerate the engraftment of the HSCs and stimulate the recovery of the blood cell–forming capacity of the marrow by directly fabricating microenvironments for each distinct lineage pathway for hematopoiesis. Indeed, the early clinical evidence showed that the added MSCs improved both the kinetics and outcomes of hematopoietic recovery in bone marrow–transplanted patients with cancer [18].

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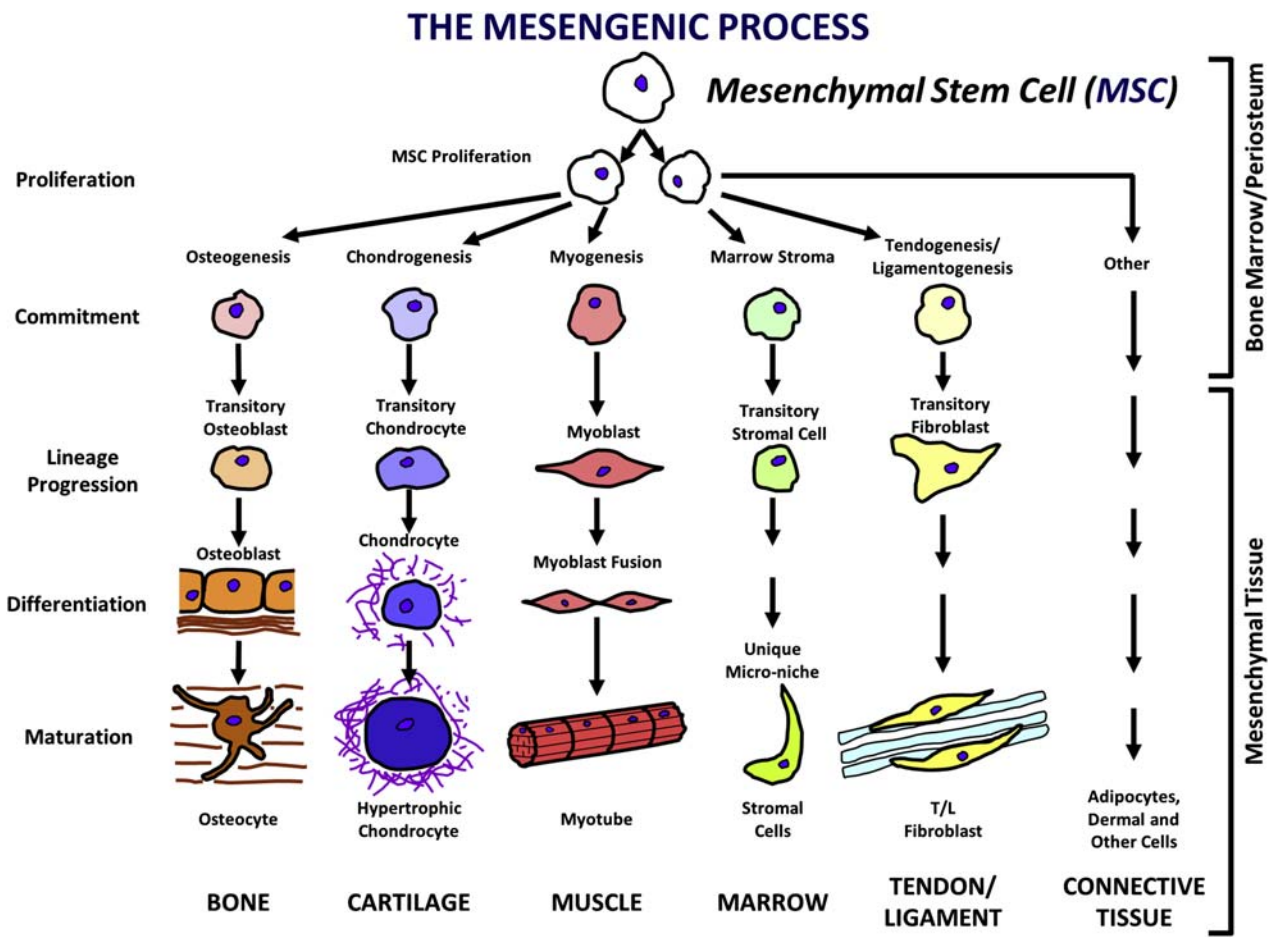

FIGURE 15.1 **The mesengenic process.** This figure was generated in the late 1980s and proposed that a mesenchymal stem cell (MSC) existed in bone marrow and that its progeny could be induced to enter one of several mesenchymal lineage pathways [5–14]. The left side of the diagram was already experimentally established [5] whereas little was known about the lineages on the right. The lineage format was constructed from what was known about the hematopoietic lineage pathway [4].

This involvement of the hematologists-oncologists led to the realization that hMSCs were immunomodulators and, thus allogeneic MSCs were not immediately interrogated by the host's immune system [17,19,20]. This allows allogeneic hMSCs to be infused into the bloodstream as a delivery modality with the cells functioning in tissue areas of inflammation or vascular damage. As discussed subsequently, this also allows hMSCs to be used in rodent models of multiple sclerosis (MS) [21,22], asthma [23,24], inflammatory bowel disease [25], graft versus host disease (GvHD) [26], etc., without rapid and intense immunorejection.

NEW INSIGHT

When Steven Haynesworth and I first started working with hMSCs, we developed monoclonal antibodies to cell-surface antigens on these cells called SH2, SH3, and SH4 [27]. Subsequently, we showed that SH2 was a unique antigen on endoglin (also known as CD105) [28] and SH3=SH4=CD73 [29]. The initial screens for these monoclonal antibodies were to their binding to hMSCs in culture and then to frozen sections of marrow plugs. It was not until years later that we realized that SH2-positive cells colocalized with the external aspects of small blood vessels in those marrow sections. Through the detailed research from the laboratories of B. Peault [30], I. Bianco [31], and others [32], we now understand that MSCs are identical to perivascular cells, referred to here as pericytes. Indeed, I have hypothesized [33] that *all* MSCs are derived from pericytes (the reverse is not correct in that some pericytes are so highly differentiated that they cannot form MSCs). In this context, we published an article on human skin showing SH2-positive cells sitting on blood vessels [34]. For reasons still not clear, only a small proportion of

such cells stained positive in sections of skin, with the most numerous positive images in specimens from young donors. Perhaps the antibody SH-2 stains differently from CD105 [28].

The current literature documents that MSCs can be isolated from marrow [35], fat [36,37], muscle [38], skin [39], periosteum [40], tendon [41], neural tissues [42], etc. These tissues all have blood vessels in common and such vessels have perivascular cells on the adlumenal surface. The positioning of such pericytes is controlled by a number of factors, but the prominent component is the platelet-derived growth factor (PDGF) receptor [43]. Thus, PDGF not only acts as a powerful chemoattractant and mitogen for mesenchymal cells, its receptors function to stabilize interactions between perivascular cells and vascular endothelial cells. I would suggest that in tissue domains of inflammation or blood vessel damage, the pericytes are liberated from their adlumenal locations and apparently function as activated MSCs.

Based on the clinically relevant effects of activated MSCs, we have suggested that the therapeutic capabilities of MSCs (i.e., liberated pericytes) reflect their biologic functionality at sites of tissue injury or inflammation. These therapeutic activities involve the modulation of the local immune cells to inhibit their surveillance of the damaged tissue, thus inhibiting the initiation of autoimmune activities [44]. Furthermore, the activated MSCs produce trophic effects by secreting a spectrum of bioactive molecules that inhibit apoptosis (especially in areas of ischemia), prevent scar formation, and stimulate angiogenesis by secreting vascular endothelial growth factor to attract endothelial cells to form new blood vessels, forming perivascular contacts stabilizing such newly formed vessels [45], and secreting mitogens that directly affect tissue intrinsic progenitors [46] (Fig. 15.2). These effects have resulted in clinically relevant therapies, as discussed subsequently and outlined in Table 15.1.

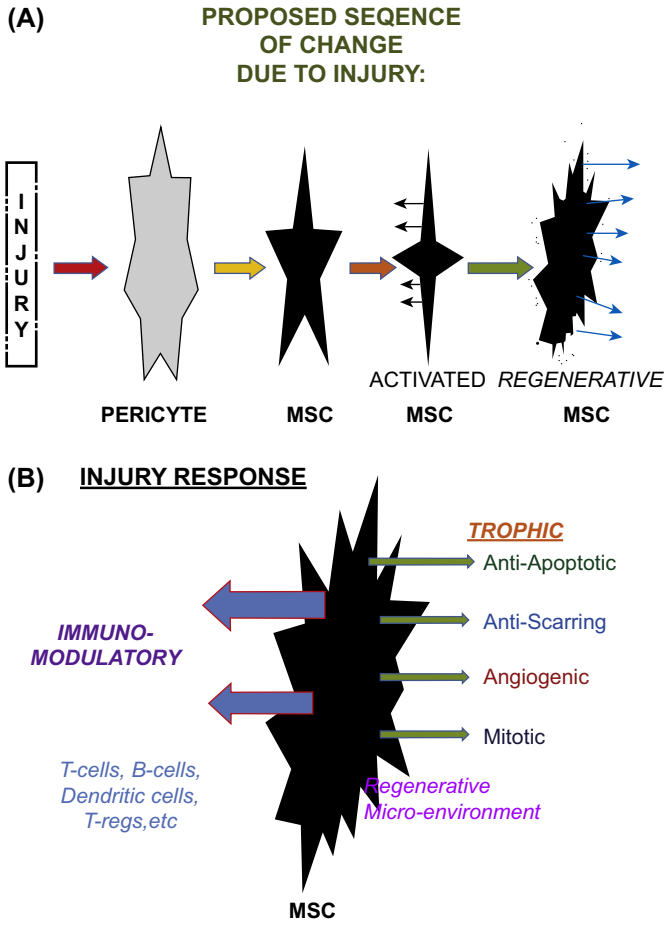

FIGURE 15.2 **Mesenchymal stem cells (MSCs) are immunomodulatory and trophic.** (A) The sequential activation as a response to injury affects pericytes and liberates them from functional contact with blood vessels to become functional MSCs. These MSCs become immuno-activated and secrete factors that organize a regenerative microenvironment. (B) The bioactive molecules secreted by activated MSCs are immunomodulatory and affect a variety of immuno-related cells [17,19,32,33]. Other secreted molecules establish a regenerative microenvironment by establishing a powerful trophic field [44,46].

TABLE 15.1 Therapeutic Effects of Mesenchymal Stem Cells

Tissue	Injury/Disease	References
Bone marrow	Graft versus host disease	[26,71]
Brain	Stroke/multiple sclerosis/ amyotrophic lateral sclerosis	[21,22,53,58,72]
Spinal cord	Cut/contusion	[73,74]
Bowel	Inflammatory bowel disease	[25,48]
Tendon	Tendonitis	[75,76]
Muscle	Defect/muscular dystrophy	[38,47]
Pancreas	Diabetes	[61]
Lung	Asthma/cystic fibrosis	[23,24]
Heart	Acute myocardial infarct, chronic	[64,65]

Given the proposed pericyte—MSC relationship, it is easy to understand why large numbers of MSCs can be isolated from adipose or muscle tissue. Indeed, although marrow MSCs are the most widely studied ones, data from fat and muscle appear to be complementary [47,48]. The fact that fat contains a 300- to 500-fold greater number of MSCs per milliliter of material accounts for the assertion that adipose-derived MSCs can be obtained in sufficient numbers without in vitro expansion [49]. Simply, liposuctioned fat is dispersed in a homogeneous slurry and incubated with digestive enzymes such as collagenase to free the pericytes from their capillary endothelial cell attachments, and the liberated cells are centrifuged after saline washes [50]. The adipocytes float and the pelleted cells, called the stromal vascular fraction (SVF), contain MSCs with some contaminating endothelial cells and monocytes [51]. For autologous cell-based MSC therapy, the SVF has been used directly with clear therapeutic effects [52,53]. Likewise, the SVF represents a relatively pure starting suspension for the culture attachment of MSCs and their further expansion.

Again, for emphasis, relatively large numbers of MSCs can be obtained from muscle [38], placenta [52] and other highly vascularized tissues [54]. From unpublished studies in my laboratory (Sung, Lennon, and Caplan), I suggest that MSCs are absent in cord blood but plentiful in the perivascular fraction of the cord. I further assert that if MSCs have been identified in cord blood, they have been found as an artifact of collection and are dislodged from their natural habitat (by needle insertion) during cord blood retrieval.

ALL MESENCHYMAL STEM CELLS ARE NOT CREATED EQUAL

Because I have asserted that *all* MSCs are derived from pericytes, it also follows that MSCs from different tissue sources must be different, because they reside in different tissue microenvironments. We have published various assays to test for the capacity of MSCs (primarily from marrow) to differentiate into bone [8], cartilage [10], muscle [12], and marrow stroma [55]. In many cases, these assays have been optimized for marrow MSCs [56], but not for MSCs from other tissues. For example, dexamethasone stimulates hMSCs to differentiate into osteoblasts but it induces mouse MSCs to become adipocytes [57]. For cartilage induction, hMSCs from marrow require transforming growth factor-β (TGF-β) in defined medium [10]. Fat-derived hMSCs do not respond to TGB-β alone but require the addition of bone morphogenetic protein-6 with TGF-β for optimal chondrogenic differentiation [58]. The relationship between the capacity of MSCs to be induced into specific and multiple phenotypic pathways and their intrinsic secretory profile remains unknown. It is clear that MSCs from a variety of tissues are multipotent, but their capacity to respond to certain signaling molecules and their ability to differentiate are apparently independent of their source or their therapeutic or secretory potential. MSCs are presumably conditioned by the tissue microenvironment from which they were purified, and thus all MSCs are not equivalent.

Clinically Relevant Therapies Using Mesenchymal Stem Cells

Often the US Food and Drug Administration requires animal trials as preclinical proof of concept for the use of a drug or device. No animal models match the human disease condition exactly. Indeed, in most cases of animal models of cell-based therapies, autologous animal cells have been preferentially used [9,13,38,41]. The reason for

including this section on preclinical studies is to set forth the unique example that hMSCs are being used in animal models to cure their disease states (see, for example, Bonfield et al. [23,24] and Bai et al. [22]). This in itself is a remarkable property of hMSCs, in that the animals' immune systems do not overtly reject the human cells whereas the (usually, second passage) hMSCs orchestrate the cures.

Multiple Sclerosis

Experimental Autoimmune Encephalitis Model

A mouse model for MS involves exposing immunocompetent animals to molecules from myelin in the presence of adjuvant that causes the animals to mount an immune reaction to these molecules, which in turn causes a series of demyelinating events mimicking human MS. Two models have been used by us: severe, monophasic MS and recurring-remitting MS. In both cases, hMSCs cure the animal after a single tail vein injection of 10^6 hMSCs [22]. We have separately shown that molecules secreted by hMSCs preferentially cause central nervous system neural stem cells to differentiate into oligodendrocytes, the cells that wrap nerve axons with myelin [21]. The end result is a cured animal that exhibits no signs of MS.

Asthma

Ovalbumin Model

Mice were injected with the chicken protein ovalbumin in adjuvant to immune-sensitize the animal. After 2 weeks, ovalbumin was atomized daily or every other day into the lungs of these sensitized mice, causing an intense inflammatory response. At 3 or 7 days or 11 weeks after the initial exposure of the lungs to ovalbumin followed by exposure to ovalbumin daily or every other day, 5–10 mice were injected in the tail vein with 10^6 hMSCs [23,24]. Ovalbumin treatment was continued for 4 days or 1 week after the injection of hMSCs; the animals were killed at 1, 4, 8, or 12 weeks after the initial ovalbumin exposure to the lungs was begun. The hMSCs were compared with saline-injected animals. In the saline-injected, ovalbumin-sensitized mice, the lung tissue was hugely inflamed; at the longer time points, scar tissue was apparent. In the hMSC-injected mice, the lungs looked normal with the reestablishment of intact endothelial lining and the absence of inflammation [23,24]. Experiments document that scar tissue from chronically inflamed lungs is replaced by normal tissue after multiple hMSC exposures [24]. Again, hMSCs facilitate the cure of this asthma-like condition in mouse models.

Inflammatory Bowel Disease

Mice can be exposed to dopamine dysregulation syndrome, which causes severe inflammatory bowel disease that is lethal. When hMSCs are targeted to the sites of inflammation using a proprietary cell-docking system, 80%–90% of the animals are cured [25]. No other single treatment can accomplish this cure. Whether the enhanced targeting is required for human subjects is not known, although phase I and II clinical trial data appear to indicate that substantial improvement in patients with Crohn disease can be experienced with infusion of hMSCs alone, with high doses required (Osiris Therapeutics, Inc., www.osiristx.com; now owned by Mesoblast, Ltd.).

Stroke and Acute Myocardial Infarct

By occluding a major artery leading to the brain [59] or suturing the heart [60], mouse models of stroke or acute myocardial infarct (AMI) have been created. Two to 7 days after these models of severe ischemia were created, hMSCs were injected via the tail vein [59,60]. The animals were monitored for 5–10 weeks and those that received hMSC therapy returned to full and normal function. The therapeutic effects resulted from the trophic activity of hMSCs that homed to sites of vascular injury and inflammation.

Diabetes

In both mice and rats, the immune destruction of pancreatic islet cells was initiated by exposing the animal to streptozotocin. By monitoring systemic secretion of insulin, a continuous, autoimmune-mediated elimination of islets, and thus the diminution of insulin secretion, was observed. Using a single intravenous injection of hMSCs, the decline of insulin secretion was halted and the eventual regeneration of islet cells was observed [61].

CLINICAL TRIALS

Worldwide, over 830 separate clinical trials are being conducted using MSCs for various clinical maladies; the most are conducted outside the United States. Such clinical trial listings do not include infusions of MSCs occurring in clinics and hospitals operating within the medical tourism industry. Indeed, the use of MSCs, both autologous (SVF and culture-expanded MSCs) and allogeneic, from marrow and other sources, form an expanding sector of the medical tourism markets in the Caribbean, Central and South America, Europe, and Asia. Most, if not all, of these MSC uses are in open-label, nonrandomized, nonplacebo controlled studies. Although one can find fault with such studies, they provide patients with access to potential therapeutics long before MSCs will be officially approved for use by regulatory agencies. Because many drugs are used "off-label" by medical practitioners, it is tempting to be enthusiastic about such treatments, especially using autologous SVF. In this regard, somewhere between 10,000 and 50,000 individuals have been infused with various preparations of MSCs with no reported adverse reactions. Certainly, if one million patients are infused with MSCs, we should expect some adverse events. In this regard, whether in sanctioned clinical trials or in other situations, the medical community must be informed quickly and completely about any and all adverse events so that we can understand the problems and limitations of these new therapies.

THE NEW MESENCHYMAL STEM CELLS

Long ago, when we developed the isolation and culturing technology and assays for hMSCs [62,63], we envisioned using these cells in tissue-engineering applications in orthopedics. We started Osiris Therapeutics, Inc. as a "bioorthopedic" company, which interestingly, followed our academic lead by furthering our initial investigator-initiated clinical trials in the augmentation of bone marrow transplantations for patients with cancer [15–19]. Within the first 10 years of Osiris, it became clear that the MSCs had a unique immunomodulatory capacity [17,19]. This led to the use of MSCs in GvHD, and the new management of Osiris brought us into the current wide range of use of MSCs as briefly outlined previously (now owned by Mesoblast, Ltd.) For the record, we set up the correct animal models for the wrong reasons. For example, the AMI model was established because it was hypothesized that MSCs would differentiate into cardiac myocytes and thus fix the damaged heart [64]. Early reports documented such differentiation by showing the labeled MSCs docked in ischemic heart tissue and the label was seen in and on cells with markers for cardiac myocytes [65]. This most probably resulted from the low frequency of fusion between the labeled MSCs and resident cardiac myocytes. We now understand more clearly that the MSCs have a complex, multicomponent trophic effect that establishes a protective and regenerative microenvironment that allows the heart to minimize the damage caused by blood vessel blockage, as shown schematically in Fig. 15.2.

The "new hypothesis" regarding MSCs is that they are site-regulated pumps for bioactive molecules that are both immunomodulatory and trophic. As outlined in review articles [44,66,67], tens to hundreds of molecules are provided and modulated at each site of injury or inflammation. Such modulation is probably site-specific and also controlled by the genetic markup of both the host and donor. The corollary is that it patients who exhibit autoimmune diseases may have defects in their MSCs with regard to specific site-regulated responses. Such defects may not be manifested by the absences of key components, but rather may be a consequence of low levels of key components. Such low levels may not be detrimental until a specific age or stress condition is experienced. Likewise, the reported decreases in MSCs with age may be controlled by the age-related decrease in tissue vascular density that is seen with age or disease state, such as diabetes. Given the relationship between pericytes and MSCs [30–33], the central issue is to determine the cause of vascular density decreases: is this controlled by the MSC/pericytes, the endothelial cells, or the tissues hosting the vasculature?

The "new" MSC, the pericyte, allows a better understanding of both a variety of diseases and the development and maintenance capacities of a variety of tissues. As a compositum of this newer information, we have constructed a poster that summarizes all of the newest information on the pericyte MSCs [68]. The new MSC treatment protocols using both culture-expanded and freshly isolated uncultured MSCs (i.e., SVF) open the door to new therapies and new medical horizons. The "old" MSCs can still be used for a variety of orthopedic indications, but they will now be used with new insights and new hypotheses. The third decade of this century will usher in "new medicine" and new treatment protocols using MSCs. The next technical hurdle will be to learn how to orchestrate the docking or targeting [25,69,70] of systemically introduced MSCs more efficiently to effect efficient therapeutic outcomes.

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CHAPTER

16

Cell Therapy of Liver Disease: From Hepatocytes to Stem Cells

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INTRODUCTION

The concept of regenerative medicine implies that the clinician works with the innate healing and regenerative process of the body to effect an improvement in a patient's health. Perhaps more than with any other organ, the liver offers the greatest opportunity for regenerative medicine. This is because, unlike most other tissues, the liver has the capacity to regenerate after massive chemical or physical insult and tissue loss [1]. Our very existence may well rely on the ability to regenerate liver mass. The liver is an incredibly complex organ that performs diverse biological functions from glycogen storage and catabolism to maintain blood sugar levels to the production and secretion of critical plasma proteins including albumin, clotting factors, and protease inhibitors. In addition, the liver is the major site in the body for the metabolism and excretion of hormones, metabolic waste products such as ammonia, as well as exogenous compounds such as toxins, drugs, and a variety of other compounds to which we are exposed through diet and environment. These processes are so critical to survival that the loss of any of these functions has serious and often lethal consequences for the individual.

Until recently, the only option for treating chronic liver disease or metabolic defects in liver function has been whole-organ transplantation. Hepatocyte transplantation has been performed as the standard. Although still an experimental therapy, there are some potential advantages for a cell therapy approach to treat liver disease. Some advantages to and problems with current treatments for liver disease are listed in Table 16.1.

Despite the unquestioned success of this technique, orthotopic liver transplantation (OLT) requires major surgery and has a significantly long recovery period. Financial costs associated with OLT and lifelong immunosuppression are considerable. There is a high incidence of complications from the surgical procedure and the concomitant immunosuppression that is required after the organ transplant. Complications can range from simple infections to renal failure, hyperlipidemia, and an increased incidence of skin and other types of cancers after long-term immunosuppression. As with all other organs, the number of liver donors does not nearly equal the number of patients on the waiting list. Patients may wait 2 or more years for a liver transplant, and there is a death rate of greater than 10% per year for patients on the waiting list. Timing is critical for whole-organ transplant. An ABO-compatible liver donor must be available when a patient requires the transplant. Some limitations associated with whole-organ transplants are addressed with hepatocyte transplants (Table 16.1). Hepatocyte transplants do not require major surgical procedures because they are performed by infusing cells into the blood supply to an organ such as the liver or spleen. Thus, hepatocyte transplants are less invasive and less costly procedures. Because major surgery is not required, fewer complications are associated with the procedure. Cell infusions are minor procedures, and thus essentially no recovery period is needed. If patients were healthy before the procedures, such as stable metabolic disease patients, they would likely feel no adverse effects from the procedure other than from the placement of a catheter. Hepatocytes can be banked and cryopreserved, so theoretically, cells could be available at any time for a patient transplant. The timing of a hepatocyte transplant depends on the status of the patient rather than on the availability of a suitable

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TABLE 16.1 Current Treatments for Liver Disease

ORTHOTOPIC LIVER TRANSPLANTATION
Major and expensive surgery
Extensive recovery period
High incidence of complications
Expensive maintenance therapy
Shortage of donor organs
Timing is critical
HEPATOCYTE TRANSPLANTATION
Less invasive and less costly procedure
Complications, fewer, and less severe
Timing of procedure is easier
Alternative cell sources
Patient retains native liver
Graft loss is not necessarily lethal
Option remains for whole-organ transplant

organ. Currently, the source of hepatocytes for hepatocyte transplants is mainly discarded organs not suitable for whole-organ transplant [2]. There are not enough hepatocytes to transplant all recipients who would likely benefit from the procedure. However, some inventive new ideas have been proposed, such as to use segment IV, which can be made available from a split-liver procedure [3] to make more hepatocytes available for transplants. Another novel source of good-quality hepatocytes is neonatal donors. Because of their small size and potential immaturity, these livers are usually not used for whole-organ transplantation. However, experience shows that these neonatal donor livers can give high-yield, good-quality hepatocytes that are resistant to cryopreservation damage. Alternative sources of hepatocytes could also be available in the future. Among the many options discussed are xenotransplants from pigs or other species, immortalized hepatocytes, and stem cell–derived hepatocytes. Future developments in these areas may make the number of cells available for hepatocyte transplants virtually unlimited.

A significant benefit of hepatocyte transplantation is that patients retain their native liver. In cases of cell transplants for metabolic disease, the patient's native liver still performs all of the liver functions with the exception of the function that initiates the disease. Patients with ornithine transcarbamylase deficiency (OTC) have a mutation in an enzyme involved in the urea cycle that prevents the metabolism and elimination of ammonia. Although the native liver is not proficient in ammonia metabolism, it is still capable of performing other liver functions including the secretion of clotting factors and albumin, drug metabolism, and all other metabolic and synthetic processes. A cell transplant only needs to support the ammonia metabolism for the patient and will not be required to provide complete liver support. Because all liver functions do not depend on donor cells, loss of the cell graft or failure of the cells to function properly will not necessarily be life-threatening, especially for a patient with stable metabolic disease. Finally, a whole-organ transplant always remains an option for the patient with a cell transplant. Even if the cell transplant fails to function or is rejected, nothing performed as part of the cell transplant procedure would likely interfere with a subsequent whole-organ transplant. Fisher et al. [4] reported that prior hepatocyte transplantation did not sensitize the cell transplant recipient to either the donor cells or to an eventual liver graft. Thus, despite sometimes transplanting hepatocytes directly into an immunological response organ (the spleen), no immunological reactions were initiated that are deleterious to the cell transplant or an eventual whole-organ transplant. However, more data must be obtained to validate these initial observations.

There are potential disadvantages of hepatocyte transplants as well. First, there are no reports of long-term complete corrections of metabolic liver disease in patients after cell transplantation alone. Because it is a new field, much additional experimentation will be required to determine the full efficacy of cell therapy of liver disease and the length of time the cell graft will function. Also, like whole-organ transplants, it is believed that cell transplant recipients will require the administration of immunosuppressive drugs. The most relevant reviews on this subject are from Jorns et al. [5], Gramignoli et al. [6], and Khan et al. [7].

BACKGROUND STUDIES

Choice of Sites for Hepatocyte Transplantation

Hepatocyte transplants have been conducted for over 20 years. A number of good reviews are available for details of the experiments and the original references that may be omitted in this review [8–13]. The large numbers of preclinical studies conducted on hepatocyte transplants firmly establish that the transplants are safe and effective. The most common sites for the transplantation of hepatocytes are the spleen and the liver; however, transplants to the peritoneal cavity, stomach, or omentum have been reported. Long-term survival of the cells is readily measured after transplants into the spleen or liver. Most cells transplanted into the peritoneal cavity intellectual property (IP) are rapidly lost. After IP transplants, only cells that nidate near blood vessels and can attract sufficient nutrition survive in the long term. Despite the ease of the procedure, IP transplants of hepatocytes have only limited efficacy. Transplants of hepatocytes to the spleen or the liver have been shown to function for the lifetime of the recipient [14–17]. Studies by Mito and coworkers clearly showed long-term survival of hepatocytes and that over time, the spleen of an animal can be "hepatized" to the point at which 80% of the mass of the organ can replaced with hepatocytes [16,18–21].

The concept of establishing ectopic liver function in the spleen is similar in theory to the bioartificial liver (BAL). In BAL, the hepatocytes are seeded into and maintained in some form of an extracorporal device. The patient's blood or plasma is pumped to the device where it interacts with the hepatocytes across membrane barriers and is then returned to the patient by a second series of pumps. There are reports that BAL can provide short-term synthetic and metabolic support [22,23]. The ease of transplant of hepatocytes and the abundance of the patient's own natural basement membrane components coupled with the naturally high blood flow make the spleen a useful site for establishing short- or long-term ectopic liver function. It is likely that hepatocyte transplants will be easier, cheaper, and more efficient, and will provide the same or better level of support as extracorporal devices.

For transplants into the liver, the preferred route for administering cells is via the portal vein. Cells are infused into the blood supply that feeds the liver and the hepatocytes are distributed to the different lobes in proportion to the blood flow they receive from the portal vein. Portal vein injections are difficult in small animals, so an alternative method is used in these studies. Hepatocytes are injected directly into the splenic pulp. The proportion of the cells that remain in the spleen is determined by the extent to which the outflow through splenic veins is impeded. In the studies of Mito et al. [16], in which the spleen was "hepatized," the authors briefly occluded the splenic outflow, which retained the cells in the spleen. Alternatively, when the spleen is used as a method to affect a portal vein injection, the splenic veins are left open. It was reported that up to 52% of cells injected into the spleen traverse to the liver via the splenic and portal veins within a few minutes [14,17].

Integration of Hepatocytes After Transplantation

Integration of hepatocytes into a recipient liver is a complex process that requires the interaction of donor and native hepatocytes to form an integrated tissue. The process may be considered to take place in four steps (Table 16.2) [24–27]. Although they are presented as separate, there is considerable overlap of the steps in both time and space. Some of the most spectacular photographs of the entire process are provided by Koenig et al. [27]. After infusion into the portal vein, hepatocytes must traverse the endothelium to escape the vascular system. Although the liver has fenestrated endothelium, under normal conditions the pores, which are in the range of 150 nm, are far too small to provide a simple transit of parenchymal hepatocytes, which are 20–50 μm in size. Infusions of hepatocytes quickly fill the portal veins and embolize secondary and tertiary portal radicals [28]. Portal pressures increase as flow is restricted by hepatocyte plugs in the portal veins. Venograms that were normal before cell transplantation

TABLE 16.2 Integration of Donor Hepatocytes Into Native Liver After Transplantation

Filling vascular spaces with donor cells
Disruption of the sinusoidal endothelium
Donor cell integration in host parenchyma
Remodeling of liver via modulation of extracellular matrix

become markedly attenuated and show greater filling of vessels proximal to the portal vein, including the mesenteric and splenic vein. If the number of hepatocytes transplanted is in the range of 5% of the total number of hepatocytes in the native liver, the portal hypertension is transient and resolves within minutes to hours.

A proportion of transplanted cells begin to fill sinusoidal spaces and the space of Disse as the endothelium in the region of the transplanted cells begins to degenerate. It is likely that both physical and humoral (growth factors and cytokines) factors are involved in this process. Microscopic analysis of tissue sections reveals that endothelium is breached in many places and donor hepatocytes leave the portal veins in regions where endothelium is incomplete and broken. Reports suggest that most hepatocytes that eventually integrate into recipient liver will have traversed the endothelial barrier by 24 h posttransplant. Cells that remain in the portal vessels are eventually removed by macrophages between 16 and 24 h posttransplant. Other reports suggest that cells may continue to integrate into parenchyma for 2–3 days after transplantation [29]. Transient hypoxia in the region of the occluded vessels leads to changes in the endothelium as well as both recipient and donor hepatocytes. Endothelium and donor and native hepatocytes all express vascular endothelial growth factor (VEGF) in the areas of hepatocyte integration [25,29], a factor known to be induced by hypoxia. It is interesting that VEGF was previously known as vascular permeability factor (VPF). Expression and secretion of VEGF/VPF, a potent angiogenesis factor, are thought to contribute to the reformation of new sinusoids and restoration of the endothelial barrier after cell transplantation.

Passage through the endothelial barrier allows donor hepatocytes to become integrated into recipient parenchyma. Full integration of donor hepatocytes and restoration of full hepatic function are difficult to ascertain. However, careful studies of the expression of antigens and activities localized to specific membrane fractions clearly demonstrate that donor hepatocytes fully integrate into the hepatic plate of native liver and for hybrid structures between native and donor cells within 3–5 days after transplantation. The antibody to CD26 recognizes the dipeptidyl peptidase IV (DPPIV) antigen, which is localized to the basolateral membrane of hepatocytes. Antibodies to connexin 32 can be used to visualize gap junctions between adjacent hepatocytes. Likewise, canicular adenosine triphosphatase (ATPase) activity can be used to identify bile canicular regions between adjacent hepatocytes. Proper localization of these different antigens and activities requires that the hepatocyte be fully integrated into the hepatic plate and polarized. By 3–7 days posttransplant, hybrid structures could be visualized in recipient liver containing both donor (DPPIV) hepatocytes and recipient ATPase activity [24] or donor DPPIV colocalized with connexin 32 [27]. Both studies clearly demonstrate proper integration of donor hepatocytes as well as the reestablishment of intracellular communication (connexin 32) between donor and recipient hepatocytes. Hybrid structures between donor and recipient hepatocytes were shown to be functional, as demonstrated by the transport and excretion of a fluorescent conjugated bile acid [24]. Hepatic transport of indocyanine and sulfobromophthalein into the bile after hepatocyte transplantation was also reported by Hamaguchi et al. [30]. Hepatocyte transplants were conducted on Eisai-hyperbilirubinemic rats. These animals have a defect in multidrug resistance protein-2, which prevents the normal transport of bile acid conjugates and their excretion into bile. This is a relevant animal model of metabolic disease because the condition is similar to Dubin–Johnson syndrome in humans. Correction of this transport defect by hepatocyte transplantation is definitive proof of the complete functional integration of donor hepatocytes into recipient liver.

As part of the integration process, there is significant remodeling of the hepatic parenchyma. Koenig et al. [27] reported the activation and release of matrix metalloprotease-2 (MMP-2) in the immediate area of donor cells. It is not clear whether the proteases are produced by the donor or recipient's cells, or even which cell type is the source of the protease, but the degradation of extracellular matrix components creates space for the donor cells and allows them to migrate from the circulation. Expression of MMP-2 was detected in and surrounding foci of proliferating donor hepatocytes 2 months after cell transplantation. Increased production and release of MMP-2 were also observed at the growth edge of nodules of fetal rat hepatocytes proliferating in adult liver after transplantation [31]. Whereas all of the components of the process are not completely understood, it is clear that hepatocytes can be transplanted into the vascular supply of the liver, breach the endothelial barrier, remodel and integrate into hepatic parenchyma, and establish communication with adjacent cells and the biliary tree, all within 3–5 days in a process of remodeling that completely retains normal host hepatic architecture.

CLINICAL HEPATOCYTE TRANSPLANTATION

Hepatocyte transplantation has been employed in clinics using three types of procedures (Table 16.3). Cell transplants were used to provide short-term liver support to patients who are dying of the disease before a suitable organ could be found. Because these patients were already listed for a whole-organ transplant, hepatocyte infusion was used, sometimes referred to as a "bridge" to transplant. A second use for hepatocyte transplants came from attempts

TABLE 16.3 Opportunities for Hepatocyte Transplantation

"Bridge" for patients to whole-organ transplantation
Cell support for acute liver failure
Cell therapy for metabolic disease

to bridge people to OLT. It was discovered that some patients receiving hepatocyte transplants recovered completely after the hepatocyte transplants and no longer required whole-organ transplant. The third general use for hepatocyte transplants is to correct metabolic liver disease. Each technique will be discussed separately.

Hepatocyte Bridge

With the bridge technique, hepatocytes are provided to a patient in acute liver failure or those experiencing acute decompensation after chronic liver disease. Most of these patients are already listed for OLT and are in danger of dying before a suitable organ can be found. Hepatocyte transplants have been conducted on these patients in an effort to keep them alive long enough to receive OLT. The primary goal of the bridge transplant is not to prevent whole-organ transplant, but rather to support and sustain the patient until an organ becomes available. Preclinical studies with several different models of acute or chronic liver failure have demonstrated that hepatocyte transplantation can support liver function and improve survival [32–41]. The results with human hepatocyte transplantation in the clinic also show an increase in the survival of patients after hepatocyte transplantation. Several reports and review articles provide details regarding patients and transplant procedures [9,11–13,42–47]. The results indicate that there is a 65% survival rate for patients receiving hepatocyte transplants. Although randomized control studies could not be conducted, preliminary results with approximately 25 patients indicate a survival advantage for patients receiving cell transplants. In addition to increased survival, there are consistent reports that clinical parameters such as ammonia levels, intracranial pressure, and cerebral blood flow improved after hepatocyte transplantation [12,42,43,46–49]. These results indicate that desperately ill patients who receive hepatocyte transplants are more likely to survive long enough to receive OLT than are the nontransplant controls.

Most patients who would be candidates for the hepatocyte bridge technique have chronic liver disease and advanced cirrhosis. Because of cirrhotic changes in the liver and accompanying portal hypertension, hepatocytes were not transplanted into the liver (portal vein) in most clinical studies. Preclinical studies were conducted where cirrhosis was induced in rats by administering phenobarbital and carbon tetrachloride [50]. When hepatocytes were subsequently transplanted into animals with increased portal pressures and cirrhosis, there was significantly greater intrapulmonary translocation of donor cells, presumably because of portosystemic shunting. These results suggest that serious complications could arise if portal infusion of hepatocytes were conducted on patients who had cirrhosis and portal hypertension. Indeed, shunting of transplanted hepatocytes to pulmonary vascular beds was reported in one clinical study [42]. To avoid this possible complication, Fisher et al. recommended that hepatocytes be transplanted into the spleen in cirrhotic patients via the splenic artery [43,47]. Despite the obvious success of the splenic artery route for hepatocyte transplantation, a report suggested that transplantation of hepatocytes by direct splenic puncture resulted in superior engraftment and fewer serious complications, although long-term engraftment was not studied [51]. Although the method for splenic delivery of cells may not be resolved, it is clear that in cases where physical and/or anatomic abnormalities are present in the native liver, the preferred route for hepatocyte transplantation is to an ectopic site, the spleen.

Promising results that have been reported suggest that hepatocyte transplantation is beneficial to patients experiencing severe hepatic insufficiency while awaiting OLT. A logical extension of these results might be to use hepatocyte transplants earlier in the process. Rather than wait until the patient is near death and with no immediate prospect for a whole-organ transplant, a more preemptive approach might be warranted. Hepatocyte transplants could be performed when patients awaiting OLT become unstable. This would presumably stabilize the patient and avoid or at least delay more serious complications of liver failure. Early intervention might avoid more costly hospitalization and other treatments.

Hepatocyte Transplantation in Acute Liver Failure

As described earlier, hepatocyte transplants have been used as a bridge to OLT. Most patients who have been referred for bridge transplants have already had chronic liver disease and cirrhotic changes in liver architecture.

There is a subgroup of patients referred for OLT who experience acute liver failure. In these patients there is massive loss of hepatocytes over a short time leading to hepatic insufficiency. Except for the dramatic loss of hepatocytes, there is no long-standing pathological change in liver architecture. Because the liver has the capacity for robust regeneration after loss of liver mass [1], there is considerable interest in trying to correct acute liver failure with hepatocyte transplantation. The hypothesis is similar to the bridge technique, in which hepatocyte transplantation is used to provide support at a time of critical and otherwise lethal liver failure. The expectation is that if the patient survives the acute loss of tissue mass, their native liver will regenerate. If the native liver regenerates, there will no longer be a need for OLT. An exogenous source of hepatocytes by transplantation would provide support of liver function to prevent lethal hepatic failure. Both donor and native hepatocytes would be expected to participate in the regeneration response. Once the native liver has been fully restored, there might not be a need for donor-derived hepatocytes. If the chimeric liver generated after the transplant is composed predominantly of native hepatocytes, the patient could safely be removed from immunosuppressive therapy. In this manner, the patient receives what amounts to a temporary liver cell transplant. If cell therapy is sufficient, the patient will be spared whole-organ transplantation and lifelong immunosuppression. Several preclinical studies support the hypothesis that hepatocyte transplantation can provide sufficient liver function to maintain an animal experiencing acute liver failure. Studies have shown that hepatocyte transplants dramatically improve the survival of animals with acute liver failure induced by D-galactosamine [37,39,40,52], 90% hepatectomy [35,36,38,53], or ischemic liver injury [41,54].

There are reports of the reversal of acute liver failure in four patients after hepatocyte transplantation [43,45,55,56]. The causes of acute liver failure ranged from hepatitis B—induced liver failure to acetaminophen intoxication, liver toxicity after eating poisonous mushrooms, and liver failure of unknown etiology in a pediatric patient. In each case, patients presented with classic symptoms of acute liver failure, and most were immediately listed for OLT. The number of cells transplanted varied for different procedures but ranged from approximately one to five billion total viable cells. In all cases, cells were transplanted into the portal vein to achieve a direct transplant into the liver. In general, patients were given fresh-frozen plasma before placement of the catheter to prevent bleeding. The results presented by Fisher et al. [55] are typical of the response to hepatocyte transplantation. There is usually a rapid fall in ammonia levels after the transplant. Circulating levels of clotting factors stabilize after the transplant and then slowly increase over the next 2 weeks. Fisher et al. reported that Factor VII levels were 1% of normal before the transplant and increased to 25% by 7 days and 64% of normal by week 2 after the cell transplant. Recovery of the clotting factors is usually rapid enough that after the cell transplant, no additional fresh-frozen plasma is required.

Patients are generally discharged within 2—4 weeks and are judged to experience a complete recovery. Cell transplant recipients ranged in age from 3 to 64 years, indicating that even older patients have sufficient regenerative capacity to be supported by hepatocyte transplantation.

As is observed with donor tissue allografts, hepatocyte allografts produce and secrete soluble human leukocyte antigen-I (sHLA-I) immediately upon implantation. If there is a mismatch between the donor and recipient, the donor-specific sHLA-I can be detected in the circulation and quantified by enzyme-linked immunosorbent assay. Donor-specific HLA class I alleles can be identified and quantified by polymerase chain reaction analysis of tissue samples taken at biopsy. When it is determined that the preponderance of cells in the patient's liver are native, the patient can slowly be removed from immunosuppressive therapy, as described by Fisher et al. [55]. In the cases that were described, patients recovered completely from liver failure after hepatocyte transplantation without serious adverse consequences and without whole-organ transplant and lifelong immunosuppression. Although the numbers of patients are small, treatment of acute liver failure by hepatocyte transplant has some significant advantages that make further investigation of this novel therapy appropriate (Table 16.3).

Hepatocyte Transplantation for Metabolic Liver Disease

A common indication for whole-organ transplantation in pediatric patients is metabolic liver disease. In these cases, there is usually a genetic defect in an enzyme or protein that is produced in the liver that inactivates a critical liver function. Although all other liver functions are generally normal, the liver is removed and replaced with a liver that can perform the missing function. Because usually only one genetic defect is associated with each metabolic liver disease, a gene therapy approach to correct the defect would seem appropriate. Unfortunately, gene therapy has met with considerable problems that have prevented successful use of this experimental technique. Hepatocyte transplantation has been used in attempts to correct metabolic defects associated with several types of metabolic liver disease (Table 16.4).

TABLE 16.4 Clinical Transplants for Metabolic Liver Disease

Familial hypercholesterolemia
Crigler–Najjar syndrome
Urea Cycle Disorders
Ornithine transcarbamylase deficiency
Carbamoyl phosphate synthetase 1 deficiency
Argininosuccinate lyase deficiency
Factor VII deficiency
Glycogen storage disease
Infantile Refsum disease
Progressive familial intrahepatic cholestasis
α1-Antitrypsin deficiency
Hereditary tyrosinemia type 1

In an approach similar to gene therapy, with hepatocyte transplants one tries to seed the patient's liver with cells that are proficient in the enzyme or function missing in the native liver. The goal is to repopulate the liver of the transplant recipient with sufficient numbers of hepatocytes to provide the missing liver function by donor cells.

Large numbers of hepatocytes cannot be infused into the portal system because of problems with embolism of the portal veins and portal hypertension. We have used as a general rule to infuse approximately 2–3 10^8 cells/kg body weight of the recipient [57,58]. Infusions of these cell numbers resulted in no long-term complications. There is always a transient increase in portal pressures that resolves within hours [45,46,57–61]. Although it is experimental, this number was arrived at by extrapolation from preclinical studies with nonhuman primates. Grossman et al. [62] reported that the infusion of 1–3 10^8 cells/kg into baboons that had previously received a left or right lobectomy was accomplished without serious complications and with only transient increases in portal pressures. Because only a few percentages of liver mass can be transplanted at any time, single hepatocyte transplants cannot be expected to replace a large percentage of liver with donor cells. For this reason, the metabolic diseases that are candidates for cell transplants are those in which the restoration of 10% of total liver function or activity is likely to correct the disease. The liver has highly redundant functions. Thus, it is recognized that 10% of a normal amount of gene product or enzyme activity would likely correct the symptoms of most metabolic liver diseases. For most metabolic liver disease and all of those listed in Table 16.4, it is believed that replacing the liver with 10% donor hepatocytes would either be completely corrective or at least ameliorate most of the symptoms of the disease.

In general, hepatocyte transplants work best when the donor cells have a selective growth advantage. There are a number of animal models of liver disease in which the native hepatocytes show an increased death rate compared with normal liver [63–66]. In these situations, when cells without the defect are transplanted into the diseased liver, the donor cells have a strong and selective growth advantage compared with the native hepatocytes. Over time the liver may become nearly completely replaced with donor cells. In certain human diseases, there might be sufficient selective pressure to favor the replacement of large parts of the liver strongly with donor cells. Such diseases include tyrosinemia type 1, Wilson disease [67], progressive familial intrahepatic cholestasis (PFIC) [63], and α1-antitrypsin deficiency [68]. In these diseases, integration of only a small proportion of liver mass by hepatocyte transplantation would likely be necessary because the donor cells would be expected to continue to proliferate in the host liver and over time replace the diseased cells. Although there are clear examples of this in studies of transplants of laboratory animals, no studies with human patients show comparable results.

Most metabolic diseases such as Crigler–Najjar syndrome (CN), OTC deficiency, and all of the diseases listed in Table 16.4 would not be expected to show such selective growth pressure for donor cells. For diseases such as these, multiple transplants over time will be required to populate the liver with 10% donor cells [69].

A large number of studies with different animal models have shown the efficacy of hepatocyte transplantation to correct metabolic liver disease (reviewed in Malhi and Gupta [10] and Strom et al. [13]. Metabolic defects in bilirubin metabolism [15,30,35,70–73], albumin secretion [16,18,69,74–76], ascorbic acid production [77,78], tyrosinemia type 1 [64], copper excretion [67,79,80], and PFIC [63], as well as other defects in biliary transport similar to Dubin–Johnson

syndrome in humans [30] have been shown to be amenable to correction by hepatocyte transplantation. These encouraging results suggested that similar defects in human patients could be corrected by hepatocyte transplantation. The diseases listed in Table 16.4 have been the focus of human trials of hepatocyte transplants.

Hepatocyte transplants were previously shown to result in the rapid correction of ammonia levels [12,42,45,47]. For this reason, urea cycle defects that result in life-threatening hyperammonemia were the first metabolic disease target for hepatocyte transplants [47,59]. In the initial study, one billion viable cells were transplanted into the portal vein of a 5-year-old recipient. Portal pressures increased from 11 cm of water before cell transplant to 19 cm immediately after the cell infusion but recovered rapidly. The patient's ammonia levels normalized without medical intervention within 48 h of cell infusion and glutamine levels returned to normal. Although OTC activity was undetectable before cell transplant, measurable OTC activity was detected in a biopsy performed at 28 days. In these studies, 10% of cells were labeled with indium^{111} before infusion into the patient to monitor distribution of the cells. Quantitative analysis of the scintigraphic images showed an average distribution ratio of liver to spleen of 9.5:1. Measurements made before cell infusion indicated that free indium was released from hepatocytes at a rate of 10% per hour, and free indium is rapidly cleared from the circulation by reticuloendothelial systems such as the spleen. Thus, most of the tracer in the spleen after cell infusion was thought to be free indium, not hepatocytes. Pulmonary radiotracer uptake was consistent with background counts, indicating the absence of portosystemic shunting despite the modest increase in portal pressures observed at the time of transplant. This first transplant for metabolic liver disease indicated that hepatocyte transplantation into the portal vein could be conducted safely in patients with no significant liver pathology with only a moderate and reversible increase in portal pressures. From the rapid normalization of ammonia levels after hepatocyte transplant, it was concluded that cell transplantation can partially correct the hyperammonemia associated with the disease. Subsequent studies verified that partial corrections of ammonia levels are possible by cell transplants alone [58,81–84]. Although complete corrections of OTC deficiency have not been accomplished, these studies indicate that cell transplants provide much-needed metabolic control of ammonia levels. Even in the absence of complete correction, liver cell transplantation should be considered a bridge to whole-organ transplantation for patients with OTC to prevent neurological problems associated with uncontrolled hyperammonemia [59,82–84].

A number of groups have attempted to correct CN syndrome type 1 with hepatocyte transplants. The first case was in typical many ways of results obtained by other groups and will be discussed in greater detail [57]. This disease is caused by a defect in the enzyme that is responsible for the conjugation and eventual excretion of bilirubin. Absence of the enzyme results in severe hyperbilirubinemia, which can lead to central nervous system toxicity including kernicterus. After the transplantation of approximately 7.5 billion cells into the liver of a 10-year-old girl, there was a slow and continuous decrease in circulating bilirubin levels over the first 30–40 days, and bilirubin conjugates were readily detected in the bile. Overall, there was approximately a 60%–65% decrease in bilirubin levels compared with pretransplant levels. Because the bilirubin conjugates could be produced only by the donor cells, their detection in the bile demonstrated the robust biochemical function of the transplanted cells and established that donor hepatocytes integrated into the hepatic parenchyma and quickly established connections with the recipient's biliary tree.

Several important findings were gained from this transplant. First, large numbers of hepatocytes could be safely transplanted into the portal vein without complication. Although the total numbers of hepatocytes in liver are difficult to assess, a transplant of 7.5 billion cells represents an estimated 3.5%–7.5% of the liver mass, which was transplanted without complication over approximately 15 h. Second, the apparent engraftment and function of hepatocytes in the clinical trials seemed to exceed that found in previous animal studies. The transplantation of 3.5%–7.5% of liver mass resulted in the restoration of approximately 5% of a normal amount of bilirubin conjugation capacity in the liver. Third, a long-term correction in bilirubin levels was observed. This patient was observed for more than 1.5 years. Fourth, single transplants of hepatocytes are effective in creating partial corrections of the disease but given the limitation of transplanting $2–3 \times 10^8$ cells/kg body weight, one cannot transplant sufficient numbers of hepatocytes to achieve complete correction of metabolic liver disease with one transplant. It is estimated that complete corrections would require two to four transplants if each were as successful and efficient as the first. Finally, this was the first unequivocal demonstration of the long-term success of hepatocyte transplantation. Although patients were bridged to transplant and clinical parameters such as ammonia levels rapidly changed after transplantation, for many of the previous patients who underwent subsequent OLT it was difficult to assess the long-term metabolic function of the transplanted cells. These studies firmly established that hepatocyte transplants were an effective means to correct metabolic liver disease. The results of hepatocyte transplants of other patients with CN largely confirm those seen with the first patient [81,85,86].

Muraca et al. [87] and Lee et al. [87a] reported partial correction of glycogen storage disease type 1 after hepatocyte transplantation. Improvement was documented by the patient's ability to maintain blood glucose between meals as well as sustained and higher glucose levels with meals. Sokal et al. [61] employed hepatocyte transplants to achieve a partial correction of infantile Refsum disease, an autosomal recessive inborn error in peroxisome metabolism of very long chain fatty acid metabolism, bile acid, and pipecolic acid. The authors reported improvement in fatty acid metabolism and a reduction in circulating pipecolic acid and bile salt levels. An overall improvement in the health of the patient was evident by the report of a significant increase in muscle strength and weight gain. Dhawan et al. [81] reported that hepatocyte transplantation partially corrected a severe deficiency in the production and secretion of coagulation Factor VII. After cell transplant, the Factor VII requirement decreased nearly 80% of that administered before heart transplantation. Stephenne et al. [88] reported the complete correction of a 3.5-year-old female patient with neonatal onset argininosuccinic lyase (ASL) deficiency. Like OTC deficiency, patients with ASL deficiency are at risk for brain damage from hyperammonemia. The patient received three sequential hepatocyte transplants over 5 months. Both freshly isolated and previously cryopreserved hepatocytes were used. At 1 year after transplant, the patient displayed 3% of normal ASL activity in hepatic biopsy samples. Engraftment of donor cells could be demonstrated by fluorescence in situ hybridization for Y chromosomes. These results confirm that hepatocyte transplantation can achieve sustained engraftment of donor cells and sustained metabolic and clinical control.

HEPATOCYTE TRANSPLANTATION NOVEL USES, CHALLENGES, AND FUTURE DIRECTIONS

Hepatocyte Transplants for Non–Organ Transplant Candidates

Most patients who have received a hepatocyte transplant were already listed for a whole-organ transplant. The need for liver support is not limited to this group. There are large numbers of patients for whom OLT is not an option. Patients in this group could include alcoholic cirrhotic patients who have not met the required abstinence period, acute liver failure patients resulting from suicide attempts, and patients with cancer. Early case reports suggested that hepatocyte transplants into the spleen could be useful to restore liver function to end-stage cirrhotic patients [12]. Although both of the patients in the reported study eventually died of concomitant renal failure that was left untreated, the patients sufficiently improved after the cell transplants to the point where they were able to be discharged from the hospital. Fox and coworkers created an animal model to study the efficacy of hepatocyte transplants to support liver function in cirrhosis in a more controlled setting. Their studies clearly demonstrated that hepatocyte transplants significantly improve liver function and survival of rats experiencing chronic liver failure after repeated injections of carbon tetrachloride [32,89,90]. Because millions of patients are currently infected with hepatitis viruses, there is clearly a need for additional means to support liver function in these patients. Notwithstanding the difficulties of such clinical studies in cirrhotic patients, cell transplantation should be thoroughly evaluated as a possible support therapy.

In addition to cirrhotic patients who may not be candidates for OLT, there are metabolic liver diseases such as phenylketonuria (PKU) that are not currently referred for OLT. Although some still believe that diseases such as PKU can be adequately controlled by diet, there is evidence of continued and progressive mental deterioration in most patients treated with diet alone. It is likely that cell therapy with hepatocytes would improve the control of phenylalanine levels in these patients. Severely affected patients with PKU and those not controlled well by diet alone should be given serious consideration for inclusion in hepatocyte transplant protocols, because it seems that the benefits would likely outweigh the risks for these individuals [91]. Reports confirm that serum phenylalanine levels were significantly reduced after hepatocyte transplants [92,93].

An important factor preventing the use of hepatocyte transplants in additional medical centers is the limited availability of hepatocytes. The normal source of cells for hepatocyte transplants are livers with greater than 50% steatosis, vascular plaques, or other factors that render the tissue unsuitable for whole-organ transplantation [2,42,45–47,55,57,58,87,94–96]. Better use of existing liver tissue could increase the numbers of hepatocytes available immediately. In the United States, no regulations require donor organs to be allocated to transplantation research centers for hepatocyte isolation, and relatively few organs go to centers where hepatocyte transplant is a possibility. Most organs that are not used for whole-organ transplant are provided to commercial firms where hepatocytes are isolated for resale or for in-house metabolism and toxicology studies. Although most uses of donor liver tissue have merit, simple allocation procedures could be instituted to route the organs to transplant centers for initial review and

selection of the most suitable cases for cell isolation. Split-liver procedures have made it possible to use caudate lobe and segment IV for hepatocyte isolation. Depending on the surgical procedure, these portions of liver tissue may remain untransplanted and have been shown to be useful for hepatocyte isolation [3]. Although currently not widely applied, most livers that are currently transplanted could be split. A portion such as the left lateral segment could be made available for cell isolation while the remaining liver tissue is used as a tissue graft. Because hepatocyte transplantation is not currently the standard of care, such proposals are not currently feasible. However, if the efficacy of hepatocyte transplants were firmly established, the risk and extra time needed for the split procedure would be outweighed by the benefit of the cell transplants. Cell transplants rather than OLT could free up the organs that are now used for acute liver failure and metabolic disease patients.

Methods to Improve Engraftment and Repopulation

Hepatocyte transplants will not be able to progress past the small numbers of patients who are being transplanted until sufficient numbers of hepatocytes become available or engraftment and repopulation are significantly improved [96]. It has become evident that pretreatment of the native liver of the transplant recipient to induce regeneration and proliferation of donor hepatocytes may be needed before hepatocyte transplantation. Most pretreatment conditioning regiments used in studies with experimental animals are too hazardous to be applied in a clinical setting. The two most common approaches that have been suggested are portal embolization and hepatic irradiation [97–99,99a]. The theories and literature on these techniques are widely available and will not be presented here. There is another technique, partial hepatic resection, more commonly called partial hepatectomy, that is commonly used in experimental animals and that has been introduced in clinical hepatocyte transplantation [86]. Because of improved techniques and instruments and increased activities at experienced centers, liver resection and living donor liver transplantation are now common procedures. With today's advanced surgical techniques and in the hands of surgeons with considerable experience with reduced grafts and split livers, partial liver resection would likely be as safe as portal embolization and hepatic irradiation and should also be considered a possible pretreatment for hepatocyte transplantation. A partial resection for tissue collection for cell isolation is a much simpler and safer surgical procedure than those routinely performed in the clinic today. Partial liver resections are most commonly performed to remove malignancies or during living donor transplant procedures. When performing a surgical procedure for tumor removal, the amount of tissue and the location of the procedure are dictated by the location of the tumor(s). Likewise, in the case of living donor liver graft removal, the surgeons need to consider preserving vessels as well as minimizing ischemia injury to the graft during surgery.

Removal of liver tissue for cell isolation will be much safer because the only concern for the surgeon will be the safety of the patient. Because the resected liver tissue will not be used as a tissue graft, the surgeon will not be concerned about the vessels in the resected tissue. Cell isolation on resected tissue can be conducted through a number of portal or hepatic vessels; thus, concern regarding maintaining appropriate hepatic artery, portal vein, and biliary flow patterns in the resected tissue is removed. The amount and exact location of the tissue to be removed can be dictated by the surgeon and performed in the safest, fastest, and easiest way. Although there are risks associated with both the surgery and anesthesia, risks associated with liver resection as a tissue source for cell isolation would likely be much lower than for living donor liver transplantation. As example, in a report of 100 donor resections from one center, no life-threatening complications occurred [100]. Hepatectomy before hepatocyte transplantation was already performed in a series of patients transplanted for familial hypercholesterolemia in 1992–1994. Those patients underwent left lateral hepatectomy to harvest tissue for hepatocyte isolation and subsequent retroviral transduction of the low-density lipoprotein receptor [101]. Transplantation of transduced autologous hepatocytes was performed on day 3 after the operation. The surgical safety of this procedure was thoroughly studied and reported to have no major complications [102].

A point that remains unanswered is to what extent hepatectomy will generate a sufficient signal to improve the engraftment or proliferation of transplanted hepatocytes. The timing of the transplantation after hepatectomy might also be important. Efimova et al. measured serum growth factors in healthy individuals after living related liver donation and showed that hepatocyte growth factor (HGF) increased 12-fold at 2 h postoperation and thereafter stabilized at a threefold higher level compared with preoperation for an additional 5 days [103]. Other growth factors such as VEGF and epidermal growth factor (EGF) did not change significantly and transforming growth factor-α (TNF-α) was not detected at all. These results were confirmed in two clinical cases of hepatocyte transplantation for CN type I. Partial hepatectomy of the left lateral section was performed immediately before hepatocyte infusion. Partial hepatectomy and hepatocyte transplantation was associated with a strong increase in serum HGF and EGF whereas TNF-α remained unchanged. These data suggest that partial hepatic resection could provide a significant

stimulus to donor hepatocytes and that the effect would last at least 5 days. Taken together, these data suggest that partial hepatic resection could be a safe and effective pretreatment to hepatocyte transplantation.

Cell Transplantation Immunology

The liver has tolerogenic properties and initially it was believed that only minimal immunosuppressive therapy was necessary in clinical hepatocyte transplantation. However, clinical experience has shown that immunosuppressive treatment is essential to avoid allograft rejection. However, there is no consensus or convincing evidence regarding the optimal immunosuppressive protocol. Most centers have adopted the same immunosuppressive protocol used for liver transplantation, including induction treatment with corticosteroids and maintenance therapy with calcineurin inhibitors, tacrolimus or cyclosporine, mycophenolate mofetil, and corticosteroids. Corticosteroids have to be used cautiously in patients with urea cycle disorders owing to their catabolic effect with a risk to induce crisis. Some centers have also added induction therapy with basiliximab or antithymocyte globulin.

Tailoring an optimal immunosuppressive treatment in clinical hepatocyte transplantation is challenging for the following reasons. No universal and reliable marker of graft function exists. In clinical liver transplantation, several simple liver function tests easily analyzed from a blood sample can exactly define liver allograft function. However, these tests are not useful in clinical hepatocyte transplantation because the hepatocyte graft usually comprises only 2%–10% of the liver mass. Furthermore, no good markers exist for allograft rejection. In clinical liver transplantation, these liver function tests are good markers of allograft rejection and histological analysis of a liver biopsy confirms the diagnosis. However, liver biopsy is not useful in hepatocyte transplantation because the procedure is invasive and has a high sampling error. Finally, knowledge is limited about the mechanism of hepatocyte allograft rejection.

Immunological studies in mouse models of hepatocyte transplantation have shown that allograft rejection is a T cell–mediated process and that humoral immunity is only minimally stimulated [104,105]. In contrast to liver allografts, which are mildly immunogenic or even tolerogenic, the allogenic hepatocytes are strongly antigenic and probably also resistant to the mechanism of allograft tolerance.

Clinical experience has confirmed that hepatocyte allograft rejection is associated with a T cell–dependent mechanism. Allen et al. [106] showed that hepatocyte allograft loss in a patient with CN type I was associated with a cellular alloresponse directed against donor class I HLA without detection of HLA antibodies. In contrast, Jorns et al. [106a] reported the appearance of de novo donor-specific HLA antibodies associated with hepatocyte allograft loss in two patients with CN. Soltys et al. [92] reported a potential noninvasive marker of hepatocyte allograft rejection. Blood analysis of allospecific CD154-positive T-cytotoxic memory cells was studied as an early marker to predict acute rejection in pediatric patients after liver, kidney, and multivisceral transplantation. In clinical hepatocyte transplantation, this assay was able to predict allograft rejection in two patients with urea cycle disorders.

Besides these mechanisms of adaptive immunity, a large proportion of transplanted hepatocytes may be destroyed by an immediate innate immune reaction. An innate immune reaction, the instant blood-mediated immune reaction, has been described in clinical hepatocyte transplantation. When in contact with blood, the expression of tissue factor on the surface of hepatocytes activates the coagulation cascade and complement system, resulting in clot formation and infiltration of leukocytes, finally destroying a major proportion of transfused hepatocytes [107,108]. Current immunosuppressive treatment protocols only partially prevent this immediate destruction. However, the addition of *N*-acetylcysteine, heparin, or low–molecular weight dextran sulfate has the possibility of ameliorating this reaction [109].

Stem Cells and Alternative Cell Sources for Liver Therapy

In addition to attempts to improve engraftment and repopulation of the liver, alternative cell sources for hepatocytes have been proposed. Xenotransplants [90], immortalized human hepatocytes [36,89,110,111], and stem cell–derived hepatocytes [112–116] and fetal hepatocytes have been proposed as alternative sources of cells for clinical transplants. To date, no alternative cell source has been found that meets all of the requirements for safety and efficacy. There is great interest in stem cell–derived hepatocytes and the possibility that they might become a future source of cells for clinical transplantation.

Proponents for the use of stem cells suggest that because of their wide availability and small size, stem cells could be a feasible and efficient alternative to hepatocytes for cellular therapy [117]. Although it is true that once generated, stem cells should be available in sufficient numbers for transplant protocols, it is not clear whether the small size of the stem cells would actually favor engraftment and repopulation of target organs. It is pleasing to speculate that the relatively low levels of engraftment of mature hepatocytes after transplant result from their large diameter, and that

the 50%–90% of transplanted hepatocytes do not engraft because they temporarily obstruct and become trapped in portal veins or hepatic sinusoids. However, obstruction of the portal vessels and hepatic sinusoids and transient increases in portal pressure may actually be necessary steps in engraftment into the liver parenchyma. Smaller, stem cell–derived, hepatocyte-like cells may actually engraft less effectively than mature hepatocytes. When examined in transplant models, smaller hepatocytes or hepatocyte-like cells were usually less effective than larger or more mature hepatocytes [13,118–120]. Sharma et al. [121] directly examined and compared the engraftment and proliferation of mouse embryonic stem cell (ES)-derived, hepatocyte-like cells and mature hepatocytes in the $FAH^{-/-}$ mouse. The $FAH^{-/-}$ is a robust model of metabolic liver disease in which transplanted donor ($FAH^{+/+}$) hepatocytes are under strong positive growth selection leading to rapid and effective repopulation of the disease mouse liver. Mouse ES-derived cells with hepatic features were found to engraft less efficiently compared with mature hepatocytes and showed limited capacity for repopulation and tissue formation.

Of the cell types most often suggested to become a source of cells for clinical transplants, ES and induced pluripotent stem cells (iPS) may hold the greatest promise for future therapy. However, to move this potential therapy to the clinics, two significant roadblocks have to be overcome: efficient and effective hepatic differentiation of the stem cells and removal of the tumorigenic potential of the transplanted cells. To date, neither condition has been met. No published protocols are efficient or effective enough to produce large numbers of mature human hepatocytes that could be used immediately for transplants. The problem of tumor formation from cells in the population that did not undergo hepatic differentiation and the possibility that differentiated hepatocyte-like cells could regress back to undifferentiated stem cells after transplantation will have to be overcome before either ES or iPS cells could be considered for clinical protocols. Also, as stated earlier, ES-derived, hepatocyte-like cells demonstrated limited capacity for liver tissue formation after transplantation compared with mature hepatocytes. Thus, much more basic research will be required before ES or iPS-derived hepatocytes are ready for the clinics.

Cell types that are currently in clinical practice and could potentially be available for cellular therapy are those from bone marrow and mesenchymal stromal cells (MSC). After the initial publication of Petersen et al., there was great excitement about the possibility that bone marrow cells might serve as a source of hepatocytes to correct liver disease [122]. Subsequent detailed work suggested that cell fusion is the principal source of bone marrow–derived hepatocytes observed in the experimental model [123]. Although there is still controversy regarding this issue, the bulk of data suggests that bone marrow is not the source of progenitor cells in the liver [124,125] and there is little evidence for the conversion of hematopoietic cells to hepatocytes in vivo in experiments with animals [126,139] or in a clinical setting [127]. When the presence of X and Y chromosomes was analyzed in liver biopsies taken from sex-mismatched liver transplant recipients (eight female to male and five male to female), the recipient-specific sex chromosome pattern was detected only in the inflammatory cells but not hepatocytes [127]. This study had a transplant to biopsy interval of 4.5 years (range, 1.2–12 years) and the authors concluded that recipient engraftment of stem hematopoietic cells is an infrequent feature in long-term grafts. Thus, bone marrow cells may not be a relevant source of hepatocytes for the treatment of liver disease.

The same cannot be said for human amnion epithelial stem cells (hAE). Whereas hAE have many of the properties that are thought to be useful in MSC, such as the ability to modulate the immune system, enhance tissue regeneration, and a lack of tumorigenicity, hAE have additional properties that make them more useful than MSC for the treatment of liver disease. Because hAE are an epithelial tissue like the liver, they do not have to cross a germ layer boundary to become hepatocyte-like cells. There is little evidence of mature liver gene expression in MSC even when transplanted into the liver, but there is good evidence that hAE cells differentiate to cells that express mature liver genes at levels that are observed in normal human liver [116], and hAE transplants have shown substantial efficacy in correcting animal models of metabolic liver disease such maple syrup urine disease and PKU [128,129]. In addition to correcting metabolic liver disease effectively, hAE reversed otherwise lethal acute liver failure in mice; and because of their immunomodulatory properties and production of HLA-G, it may be possible to transplant hAE cells into patients without using immunosuppressive drugs [129,130].

MSC isolated from a variety of tissues including cord blood, skin, and human liver have been proposed as a source of hepatocytes for transplantation. There are numerous reports of mesenchymal cells adopting hepatic features when cultured under specific conditions or upon transplantation in vivo. Several groups have reported the expression of hepatic genes and proteins normally expressed in the liver such as albumin, α1-antitrypsin, α-fetoprotein, fibrinogen, glycogen, and even some more mature hepatic markers such as drug-metabolizing genes including CYP3A4 [131–134]. In all cases, when the level of expression of hepatic genes and their functions was measured it was low compared with authentic human hepatocytes. The use of MSC as a source of hepatocytes may depend on their ability to differentiate to cells with a mature hepatic phenotype upon transplantation. When examined

carefully, after transplantation into the mouse liver human cord blood mononuclear cells resulted in small clusters of hepatocyte-like cells that expressed human albumin and Hep Par, a marker protein found in hepatocytes; however, the cells also expressed mouse cytokeratin 18, which suggested that the clusters of hepatocyte-like cells were the result of cell fusion with endogenous mouse hepatocytes [135]. There is not yet convincing evidence that MSC can differentiate to cells with a broad range of mature hepatic functions.

Although there is little substantial evidence that bone marrow or MSC from different sources form mature hepatocytes in vitro or in vivo, there is growing evidence that these cells may improve liver function when they are infused into patients with cirrhosis. A remarkable article by Sakaida et al. reported that the intravenous transplantation of bone marrow cells reduced liver fibrosis in a model of cirrhosis induced by treating mice with chemokine ligand 4 [136], which eventually led to the proposal to use autologous bone marrow therapy for liver cirrhosis [137]. Several groups soon began phase I safety and feasibility studies with bone marrow cell infusions in cirrhotic patients. Most studies have been uncontrolled investigations of the infusion of bone marrow−derived mononuclear cells [138,139] or CD34-selected cells [140−144]. In most of the studies, granulocyte−colony-stimulating factor (G-CSF) was used to mobilize CD34-positive cells. Cells were delivered through a peripheral vein or infused directly through a hepatic artery. The most common findings were a slight improvement in liver function as measured by a small decrease in bilirubin levels, which was usually accompanied by a small increase in serum albumin levels. Improvements in the Child−Pugh and/or the Model for End-Stage Liver Disease (MELD) scores were also frequently reported [140,142−144]. In one study, bone marrow−derived MSC rather than CD34-positive or unfractionated bone marrow mononuclear cells were infused via a peripheral vein, with similar results [145]. The authors observed an increase in mortality and other complications when the MSC were infused into the hepatic artery of patients with decompensated cirrhosis rather than via a peripheral vein.

In a controlled trial, a minimum of 1×10^8 mononuclear cells from bone marrow aspirates (without G-CSF pretreatment) were infused through the hepatic artery of patients with cirrhosis [146]. Fifteen patients were randomized to each arm of the study. The results indicated that the Child−Pugh score improved in the cell therapy group relative to the controls. The MELD score remained stable in patients receiving the cell therapy, whereas in the control group, the MELD score increased. Serum bilirubin levels were also improved in the treated group. The improvements noted in the different end points were significant for only 90 days. The clinical findings suggested that slight improvements in hepatic function as measured by bilirubin and albumin levels, MELD, or Child−Pugh scores were obtained after the transplantation of bone marrow mononuclear cells or partially purified CD34-positive cells. It is comforting that similar findings were obtained with different protocols and by different groups. What is not clear from these initial studies is the most useful cell type to transplant and the best route of delivery. Perhaps sustained improvements in liver function could be obtained if these parameters were optimized.

With the exception of bone marrow or bone marrow−derived stem cells, other stem cell sources have not been employed to treat liver disease. Much more research will have to be conducted before cell types such as ES or iPS could be approved for clinical trials. Adult stem cells do not seem to show sufficient engraftment, proliferation, and differentiation to hepatocytes to warrant clinical trials with any of the cell types. Human amnion epithelial cells have shown a substantial ability to differentiate to hepatocyte-like cells and correct metabolic liver disease and acute liver failure and are being translated to the clinic to treat patients. However, until hAE are approved for transplant, authentic hepatocytes remain the preferred cell type to treat liver disease. Future work with hepatocyte-based therapy will need to focus on improving the engraftment and/or proliferation of donor cells posttransplant. Even two- to fourfold increases in liver repopulation by hepatocytes over the levels obtained with current transplant procedures could lead to substantial improvement in the clinical outcome of patients with liver-based metabolic diseases. It is likely that incorporating preconditioning regimens with hepatic resection [86], ischemia-reperfusion injury, and/or radiation-induced blockage of the growth of native liver [92] will provide the selective growth advantage to the donor cells required to attain the levels of liver repopulation required to normalize alterations observed in patients with metabolic disease. Because these types of studies are being conducted at medical centers around the world, the efficacy of these modified protocols should soon be apparent.

SUMMARY

Hepatocyte transplantation studies conducted in animal models of liver failure and liver-based metabolic disease have proven to be safe and effective means to provide short- or long-term synthetic and metabolic support of liver function. For certain organ transplant candidates such as those with metabolic liver disease, cell transplantation alone could provide relief of clinical symptoms. Cell transplant studies in patients with acute or chronic liver failure

or genetic defects in liver function clearly demonstrate the efficacy of hepatocyte transplantation to treat liver disease. In virtually all cases, a clinical improvement in the condition of the patient could be documented. Although all of the initial reports concerning hepatocyte transplants are encouraging, it must be realized that there are still no reports of long-term and complete corrections of any metabolic disease in patients. The report of a complete correction of a patient with a urea cycle defect is encouraging; however, the length of time that human hepatocytes will function after transplantation has not been determined. Studies in animal models of liver disease have documented that donor hepatocytes transplanted into the spleen or the liver function for the lifetime of the recipient and participate in normal regenerative events. Although it is likely that human hepatocyte transplantation will result in lifelong and normal function of donor cells, this needs to be clearly demonstrated in a clinical study.

Future work will have to be conducted to establish optimal transplant and immunosuppression protocols to minimize complications and maximize engraftment and function. A major problem for clinical hepatocyte transplant is the inability to track donor cells after transplantation. Except for the short-term tracking of hepatocytes prelabeled with radioactive substances such as indium^{111} [59], and following differences between donor- and recipient-secreted HLA [55], there are no reports of quantitative and facile methods to detect donor cells. Relatively noninvasive methods will be needed to optimize transplant and immunosuppressive protocols as well as for daily monitoring of the cell graft. None of the problems cited here seem insurmountable. There are reports of successful hepatocyte transplants from laboratories in many different countries. The cooperative spirit that has developed among investigators in the different transplant centers should benefit the research field and especially future recipients of hepatocyte transplants.

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CHAPTER

17

Cardiac Stem Cells: Biology and Therapeutic Applications

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The heart, which was previously considered a prototypic terminally differentiated and postmitotic organ, is now known to retain reservoirs of undifferentiated cardiovascular precursors and mitotic cardiomyocytes throughout adult life [1,2]. Multiple classes of precursor cells with the ability to contribute to adult cardiomyocyte turnover have been described, including postnatal remnants of bona fide embryonic heart lineages [3–5], multipotent circulating cells of noncardiac lineage [6], and adult cardiovascular progenitors generated de novo by reprogramming mature cardiac cells to a multipotent state [7,8]. The cardiac precursor and cardiomyocyte mitosis pathways remain fully operational up to the neonatal stage before becoming idle and limited in their ability to support regeneration in response to damage [9–12]. Cell-based therapeutics offers a promising strategy to overcome this limitation and induce regeneration in the diseased heart. It falls into two basic categories: (1) direct remuscularization and (2) stimulation of endogenous repair. In the first category, engraftment of billions of laboratory-grown cardiomyogenic cells derived from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) is tested as an approach to remuscularize and revascularize a myocardial scar directly. In the second category, transplantation of cardiac and extracardiac adult multipotent stem cells aims to stimulate endogenous mechanisms of repair, which include the upregulation of cardiac precursors and cardiomyocyte mitosis, to induce regeneration. In this chapter, we review the biology of cardiac myogenesis in relation to cell-based therapeutics.

DEVELOPMENT OF THE HEART FROM CARDIAC STEM/PROGENITOR CELLS

Mammalian cardiogenesis involves the synchrony of function among a diverse population of cells that comprise the heart: atrial/ventricular cardiomyocytes, smooth muscle cells, endothelial cells, epicardial cells, conduction system cells, autonomic nerves, valvular components, and connective tissue [13,14]. The specification and differentiation of these cell lineages depend on spatially and temporally controlled developmental steps [15,16]. Four cardiac anlagen (the primary heart field [FHF], secondary heart field [SHF], and proepicardial organ [PEO], which are of mesodermal lineage; and the ectodermal lineage-derived cardiac neural crest [CNC]) are the principal sources of cardiovascular progenitor cells. Essentially, they are responsible for the development of the various cardiac structures [17–21].

The FHF and SHF lineages are specified before gastrulation, in the posterior epiblast of the early primitive streak embryo [22,23]. The epiblast, or primitive ectoderm, is the primitive cell layer from which all three germ layers (ectoderm, mesoderm, and endoderm) are derived during gastrulation. The primitive streak is the grooved structure that forms at the most proximal region of the posterior midline of the epiblast. The cardiogenic mesoderm gastrulates as the precardiac cells from the posterior epiblast ingress through the primitive streak [22,23]. This process involves the epithelial-to-mesenchymal transition of precardiac epiblast cells, mediated by the transient induction of the T-box transcription factors Brachyury and Eomesodermin, followed by expression of the mesoderm posterior 1 (*Mesp1*) gene. Precursors of the cardiogenic mesodermal progenitors express Mesp1, but the expression of this gene is downregulated upon formation of the cardiac crescent. At this stage in development, progenitors

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irreversibly commit to the cardiac lineage, expressing the LIM-homeodomain transcription factor NK2 transcription related, locus 5 (*Nkx2-5*) [22].

A tetracycline-inducible, genetic lineage-tracing approach in mice carrying a Mesp1-rtTA transgene indicates that the FHF originates from an early wave of Mesp1$^+$ progenitors in the anterior splanchnic mesoderm and forms the Nkx2-5$^+$ cardiac crescent, from which the cells migrate medially to form the linear heart tube [24]. Ultimately, the FHF contributes to left ventricle and atria formation [17,21,25]. The SHF originates from a late wave of Mesp1$^+$ progenitors in the pharyngeal mesoderm medial to the cardiac crescent and lies anterior and dorsal to the linear heart tube. Ultimately, the SHF contributes to the right ventricle, outflow tract, and atrial tissue formation, accounting for two-thirds of the cells in the heart [17,21,25]. Although not all genetic markers unique to the FHF and SHF have been identified, some transcription factors and signaling molecules are known to characterize each population. Both are marked by Nkx2-5, but the FHF progenitors are distinguished by T-box transcription factor Tbx5 and basic helix-loop-helix transcription factor Hand1, whereas the SHF progenitors are distinguished by Hand2, Isl1, and Fgf10 [17,26,27]. Although a retrospective clonal analysis in the mouse embryo suggested the FHF and SHF progenitors originate from a common precursor [25], further investigations must be pursued to determine whether the FHF and SHF progenitors stem from a single precursor or perhaps a subset of precursors. As mentioned previously, the FHF and SHF are governed by both shared and distinct genetic programs. Overall, Nkx2-5 and GATA transcription factors mark the FHF progenitor population and Isl1, Foxh1, and Mef2c mark specialization of the SHF progenitors. However, the transcriptional control that comes with the lineage segregation is still uncertain.

The PEO organ, a cauliflower-like structure that arises at the inflow region of the looping primordial heart [28–30], arises from an early wave of Mesp1$^+$ progenitors, which are thought to be distinct from the FHF/SHF-forming Mesp1$^+$ cardioblasts [24]. Whether the Mesp1$^+$ cells of PEO lineage are also prespecified in the epiblast is not known [23]. The PEO progenitors will ultimately develop into the epicardium, a layer of cells that delineate the outer layer of the primordial heart and contribute to the development of the coronary vasculature, myofibroblasts, and other epicardial cells [31–34].

CNC cells are a population of vertebrate-specific, extracardiac, migratory multipotent progenitor cells of neuroectodermal lineage (Mesp1-negative), which arise transiently from rhombomeres 6 and 7 at approximately Carnegie stage 12 in humans [35]. The role of CNC cells in mammals is diverse. Part of their function is to contribute to cardiac and aorticopulmonary septation, valvulogenesis, and autonomic neurogenesis [36]. The traditional view is that neural crest cells are specified during neurulation through an epithelial-to-mesenchymal transition at the roof plate of the neural tube, the site where the surface ectoderm converges with the neural ectoderm. However, lineage-tracing experiments in chick embryos suggest that, similar to the mesoderm, specification of the neural crest lineage is independent of neurulation and starts before gastrulation in the epiblast, bilaterally to the Hensen node, about 300 μm from the primitive streak [37]. Intriguingly, transplantation of quail-donor Hensen node cells to precardiac and nonprecardiac regions of chick host epiblast indicates that neural crest cells may contribute to cardiogenesis [22]. Similarly, fate-mapping studies in fish and mice also indicated that about 10% of cardiac myocytes are developmentally derived from the CNC [3,38–40].

CARDIAC STEM/PROGENITOR CELLS IN THE ADULT HEART

Although the adult heart was traditionally thought to be a postmitotic and terminally differentiated organ, a number of studies indicate that cardiac myocytes regenerate continuously throughout the adult life, albeit at a seemingly slow pace (Fig. 17.1) [1,2,41]. Cardiomyocyte renewal occurs either via division of preexisting cardiomyocytes [1,41,42] or via pools of undifferentiated cardiac progenitor cells [41,43,44]. Some of these progenitor cell types are thought to be remnants of the embryonic cardiac progenitor cell programs, although their exact lineage relationships and plasticity remain unclear.

c-Kit$^+$ Cardiac Progenitor/Stem Cells

Beltrami and colleagues provided the first evidence of cardiac progenitor/stem cells residing within the adult heart [45,46]. These investigators described niches of stem/progenitor cells within the adult heart, identified by the expression of the proto-oncogene c-Kit on their cell surface [45,46]. c-Kit$^+$ cardiac stem cell (CSC) niches reportedly reside throughout the ventricular and atrial myocardium but have a higher density in the ventricular

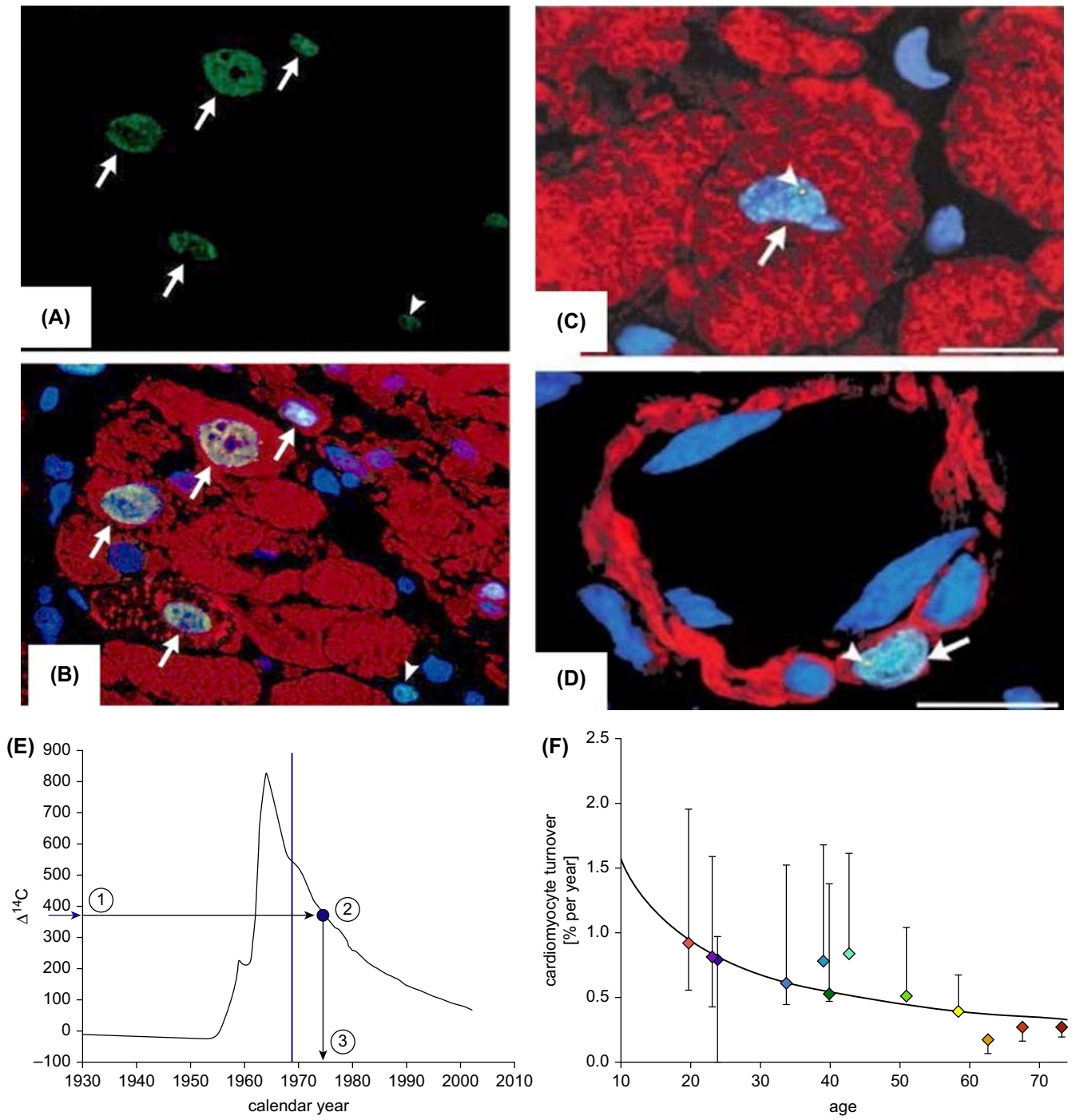


FIGURE 17.1 **Cardiomyocyte renewal in adult humans.** (A, B) By analyzing the presence of the cell proliferation marker Ki67 in adult human hearts, Beltrami and colleagues provided strong evidence that human cardiomyocytes divide throughout adulthood and in response to ischemic damage. Arrows point to cycling cardiomyocytes in an infarcted human heart, identified by confocal immunofluorescence for Ki67 and the cardiomyocyte marker α-sarcomeric actin. (C, D) By examining postmortem cases in which male patients received heart transplants from female donors, Quaini et al. detected a high level of cardiac chimerism caused by the migration of Y chromosome–bearing primitive cells from the recipient to the grafted heart. Putative stem cells and progenitor cells were identified in control myocardium and in increased numbers in transplanted hearts. Arrows point to a Y-chromosome^{+} cardiomyocyte (C) and coronary vascular cell (D) in a female heart transplanted into a male recipient. (E, F) Bergmann and colleagues took advantage of the integration of 14C, generated by nuclear bomb tests during the Cold War, into DNA to establish the age of cardiomyocytes in humans (E), and found that cardiomyocytes renew, with a gradual decrease from 1% turning over annually at the age of 20% to 0.3% at age 75; overall, less than 50% of cardiomyocytes are exchanged during a normal life span (F). These results support the original observations by Beltrami et al. and Quaini et al. *(A, B) Adapted from http://www.nejm.org/doi/full/10.1056/NEJM200106073442303#t=article; (C, D) Adapted from http://www.nejm.org/doi/full/10.1056/NEJMoa012081#t=article; (E, F) Adapted from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2991140/.*

apex. c-Kit$^+$ cardiac progenitors do not express transcription factors or membrane and cytoplasmic proteins that may characterize them as bone marrow (BM), neural, skeletal, skeletal muscle, or cardiac cells. When isolated and cultured ex vivo, c-Kit$^+$ CSCs proved to be self-renewing, clonogenic, and multipotent, developing into cardiomyocytes, endothelial cells, and smooth muscle cells. More important, transplantation of culture-expanded c-Kit$^+$ CSCs results in their multilineage differentiation into cardiomyocytes and endothelial and smooth muscle cells, providing a potentially important cell-based therapeutic strategy for promoting heart regeneration in patients with heart disease [43,45–47]. Interestingly, genetic linage fate-mapping experiments in mice suggest that c-Kit$^+$ cardiac cells may not represent a common, multipotent cardiovascular stem cell, as was originally suggested [46], but distinct classes of cardiovascular cells with restricted lineage differentiation potentials [15]. For example, fate mapping of *c-Kit* expression in the heart indicates that most adult c-Kit$^+$ cardiac cells are endothelial lineage-committed cells (either undifferentiated endothelial progenitor cells [12,48,49] or fully differentiated coronary endothelial cells [12,50]), whereas only a relatively small fraction of c-Kit$^+$ cardiac cells represent cardiomyocyte progenitors [3,12,49,51]. *c-Kit* expression has also been reported to be retained in a small subset of differentiated adult cardiomyocytes [12,48,50,52]. However, other studies have failed to confirm expression of c-Kit in the coronary endothelium [3,8,51] and differentiated myocardium [3,49,51]. Temporal analyses of c-Kit expression during mouse cardiogenesis using tamoxifen-inducible *Cre*-reporter mouse lines provide compelling evidence that cardiomyogenic c-Kit$^+$ cardiac progenitor cells are derived from the CNC, whereas a vasculogenic c-Kit$^+$ cardiac cell originates in the cardiogenic mesoderm [3,10,15,50]. The origin of the c-Kit$^+$ cardiomyogenic progenitor pool in the CNC may partly explain their limited prevalence and contribution to the myocardial lineage [3,15]. iPSC models of cardiogenesis suggest that activation of canonical bone morphogenetic protein (BMP) signaling in the outflow tract of the mouse embryo during the invasion of CNC cells is a limiting factor for c-Kit$^+$ CNC progenitors, because transient BMP antagonism extensively induces their in vitro differentiation into beating cardiomyocytes (Fig. 17.2) [3].

Isl1$^+$ Cardioblasts

Following the seminal observation of adult c-Kit$^+$ CSCs by Beltrami and colleagues [46], Laugwitz and colleagues identified through *Isl1-Cre* reporter mouse line approaches the retention of a multipotent Isl1$^+$ progenitor cell pool in the postnatal mouse and human heart [4]. Isl1$^+$ cardioblasts are reportedly distinct from c-Kit$^+$ CSCs based on the lack of c-Kit antigen expression [4]. They have been isolated and expanded from embryonic and postnatal hearts and have been shown to differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells, among others [4,53–55]. Based on the original observation that Isl1 is specifically expressed in SHF progenitors [26], postnatal Isl1$^+$ cardioblasts are thought to comprise remnants of the SHF program [53]. However, subsequent studies demonstrated that both SHF and CNC cells express Isl-1 at a stage-specific manner [3,56–59]. Other studies suggested that a subset of Isl1$^+$ cardiomyogenic progenitors in the postnatal mouse heart may be PEO derivatives [5,30,60]. However, this hypothesis has been challenged by subsequent work indicating the lack of cardiomyocyte differentiation capacity of epicardial progenitors [31,33,34,61]. Similar to adult c-Kit$^+$ CSCs [49,50], postnatal Isl1$^+$ cardioblasts minimally contribute to adult heart regeneration [59]. Nonetheless, the unequivocal presence of multipotent Isl1$^+$ cardiac progenitors in the adult heart has major implications for the prevention and treatment of congenital and adult heart diseases; therefore, further studies are warranted for delineating their developmental origin(s) and the molecular mechanisms regulating their expansion and cardiovascular fate commitment.

Cardiac Neural Crest–Derived Progenitors

Given its naturally limited role in cardiomyogenesis, one of the most intriguing and unexpected functions of the neural crest lineage in the heart is its contribution to postnatal cardiac progenitor cells. Tomita and colleagues used a neural crest–specific myelin protein zero (P0) *Cre*-reporter mouse line to explore the potential contribution of the neural crest in postnatal cardiac progenitor cells [40]. The investigators showed that a portion of *P0-Cre* derivatives in the postnatal heart are multipotent neural crest progenitors that, when isolated and expanded in vitro, could develop into peripheral nervous system-type neurons, Schwann cells, smooth muscle cells, and cardiomyocytes. Notably, some of these neural crest–derived progenitors reportedly express c-Kit at low levels and share common characteristics with other putative stem/progenitor cell types that have been reported in the adult mouse heart, such as the Abcg2$^+$ side-population (SP) cells [62] and cardiosphere-forming cells [9,63]. This suggests that most adult cardiac progenitor cell types, with the possible exception of Sca-1$^+$ progenitors [64], which are discussed

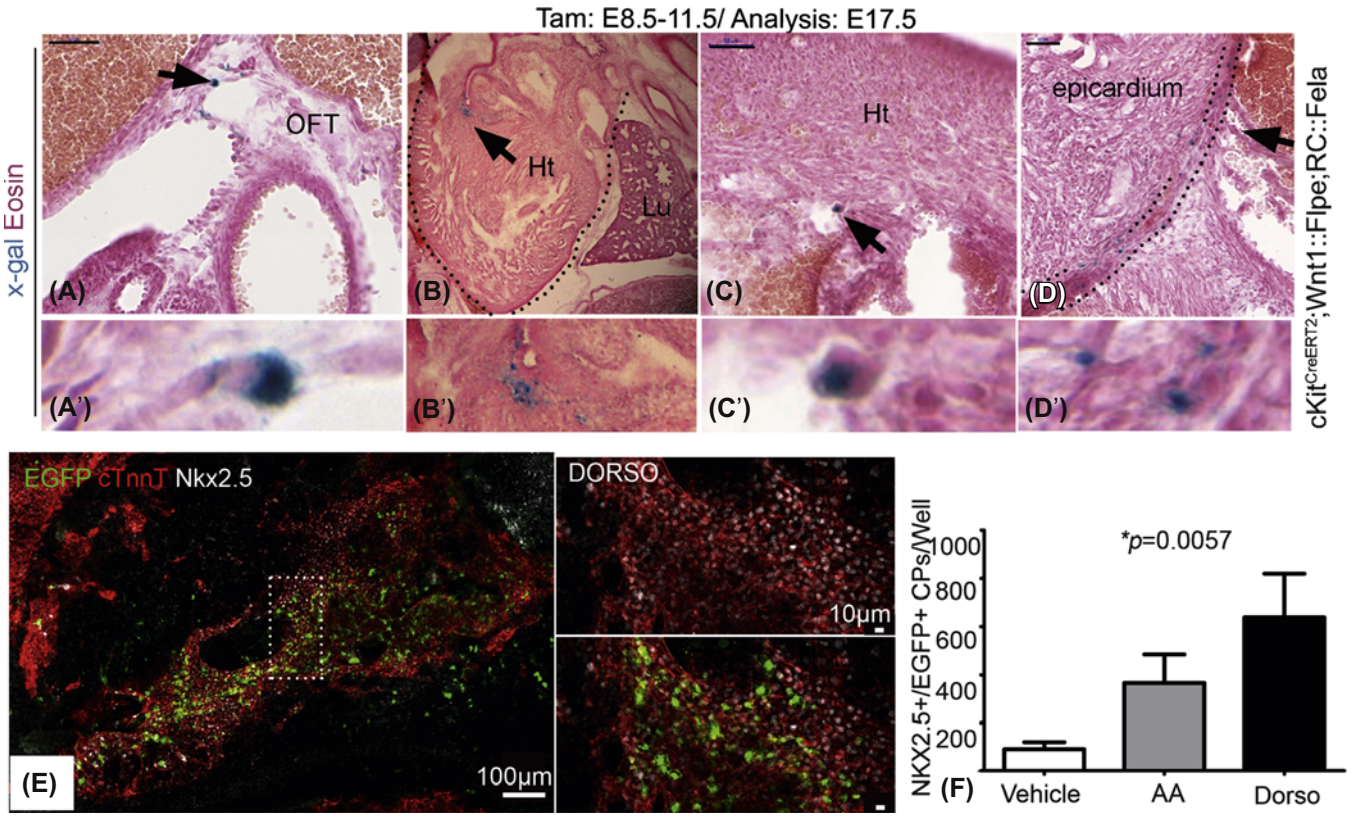


FIGURE 17.2 The cKit receptor is expressed on Nkx2.5^{+} cardiomyogenic cells of neural crest lineage whose contribution to cardiomyogenesis is limited by bone morphogenetic protein (BMP) signaling. (A, A′–D, D′) An intersectional genetic fate-mapping strategy with *Wnt1::Flpe and cKit-CreERT2/+* alleles allowed the lineage tracing of neural crest–derived cKit^{+} cells in the heart. Expression of the reporter gene β-galactosidase was analyzed via X-gal staining and indicated the presence of a limited number of neural crest–derived cKit^{+} progenitors in the outflow tract (A, A′), atrioventricular junctions (B, B′), ventricular myocardium (C, C′), and epicardium (D, D′). (E, F) An in vitro model of cardiogenesis with *cKit-CreERT2/+* reporter-induced pluripotent stem cells further suggested that the cardiomyogenic contribution of cKit^{+} neural crest progenitors is significantly enhanced when the canonical BMP pathway is transiently inhibited with the BMP antagonists noggin or dorsomorphin. Magnifications: 50 μM in A–D; 10 μm in E; and 10 μM in right two "DORSO" panels of E. *Adapted from Hatzistergos KE, Takeuchi LM, Saur D, Seidler B, Dymecki SM, Mai JJ, White IA, Balkan W, Kanashiro-Takeuchi RM, Schally AV, et al. cKit+ cardiac progenitors of neural crest origin. Proc Natl Acad Sci USA 2015;112:13051–6. https://doi.org/10.1073/pnas.1517201112.*

subsequently, may share a common neural crest origin. Subsequent studies using *Wnt1-Cre* [65] and *Kit-CreERT2/+* reporter mice [10] have corroborated the presence of cardiomyogenic neural crest–derived progenitors in the postnatal heart and raised the possibility of mobilizing them therapeutically in response to heart damage either pharmacologically, by activating the monocyte chemoattractant protein-/CCR2 axis [65], or by activating the stromal cell–derived factor 1 (SDF1)/CXCR4 axis via transplantation of adult BM–derived mesenchymal stem cells (MSCs) [10] (Fig. 17.3).

Epicardial Progenitor Cells

The epicardium is the cardiac structure with perhaps the highest proliferative and regenerative capacity after birth. Both mammals and fish vigorously activate epicardial cells via epithelial-to-mesenchymal transition in response to damage [66,67]. A genetic fate-mapping study in mice showed that compared with the adult myocardium, which holds minimal regenerative capacity, the adult epicardium requires about 3 days to regenerate itself fully in response to ischemic damage [67]. This dynamic epicardial injury response led to the original hypothesis that the epicardium is the primary postnatal source of multipotent cardiovascular progenitors for myocardial lineage regeneration in the adult heart [5,66,68]. However, subsequent studies in fish [61], mice [31,33], and humans [32,34] provided compelling evidence suggesting that the epicardial lineage is unlikely to contribute myocardial cells during cardiac development, homeostasis, or regeneration. Nonetheless, the adult epicardium is invested with undifferentiated progenitor cell pools that are generally identified in mice by the

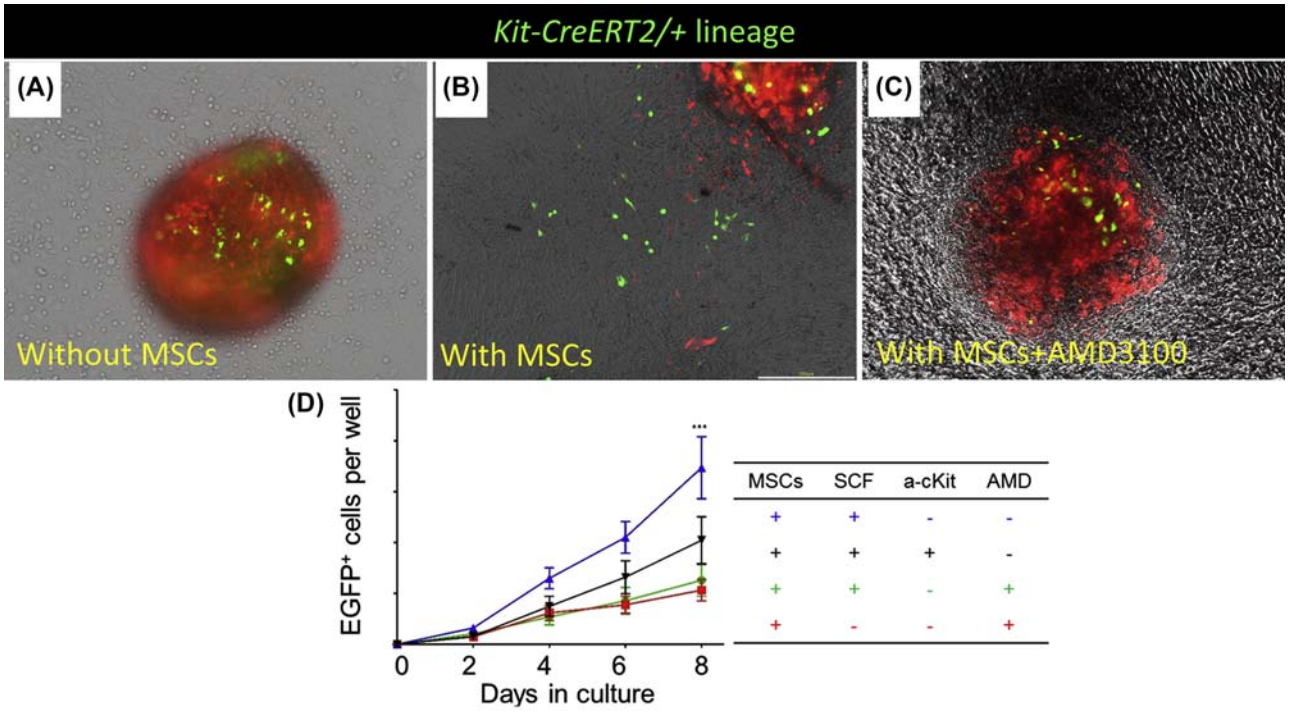


FIGURE 17.3 Regulation of cardiac cKit$^+$ cell (CSCs) self-renewal by mesenchymal stem cells (MSCs). (A) A tamoxifen-inducible *Kit-CreERT2/+;IRG* mouse line enables real-time imaging of CSCs based on *Cre*-mediated labeling with an enhanced green fluorescent protein (EGFP) reporter gene. Live-tissue fate-mapping analysis of EGFP plus CSCs in cultured myocardial explants indicates that migration and proliferation of CSCs are significantly impaired in the postnatal heart (A–D). (B) Coculture with MSCs stimulates proliferation and migration of CSCs from within the myocardial explants into the MSC culture (B–D). (C) The chemotactic effects of MSCs are blocked by the SDF1/CXCR4 inhibitor AMD3100 (C, D). These findings support the hypothesis that MSCs stimulate the proliferation and migration of CSCs via the SDF1–CXCR4 axis. *SCF*, stem cell factor. *Image adapted from Hatzistergos KE, Saur D, Seidler B, Balkan W, Breton M, Valasaki K, Takeuchi LM, Landin AM, Khan A, Hare JM.. Stimulatory effects of MSCs on cKit+ cardiac stem cells are mediated by SDF1/CXCR4 and SCF/ckit signaling pathways. Circ Res September 30, 2016;119(8):921–30. https://doi.org/10.1161/CIRCRESAHA.116.309281.*

expression of the cell-surface markers platelet-derived growth factor receptor-α (PDGFRα) [60] and/or stem cell antigen-1 (Sca-1) [69,70]. Intriguingly, adult PDGFRα$^+$/Sca-1$^+$ epicardial progenitors exhibit MSC-like properties, based on their ability to form colony-forming unit-fibroblasts and differentiate into osteogenic, adipogenic, and chondrogenic derivatives ex vivo. Other markers, such as the transcription factors Wilms Tumor 1 (WT1) and Tbx18, are also reported to be coexpressed in multipotent adult epicardial progenitors [5,60]. Adult epicardial progenitor cells have an important role in adult heart regeneration, either directly by contributing to coronary vessel, epicardial adipose tissue, and myofibroblast regeneration, or indirectly through paracrine mechanisms that support cardiomyocyte survival and proliferation [71,72].

CELL-BASED THERAPEUTICS FOR HEART DISEASE

The most important unmet need in cardiovascular medicine is that of a regenerative therapy. Although the heart has regenerative capacity, it is limited; and ischemic and other types of cardiac injury leave permanent injury and impairment in heart function, which in turn produce the major burdens of morbidity, mortality, and health care costs. As such, there is major impetus to translate the new knowledge of CSC biology into the therapeutic arena (Fig. 17.4). There have been attempts at cell-based therapy using both cardiopoietic cells (either endogenous adult CSCs or pluripotent stem cell–derived cardiac cells) and cells derived from other body sites, most notably BM [73,74].

Mortality rates from ischemic heart disease are falling, but paradoxically the incidence of heart failure (HF) is a growing cause of morbidity and mortality worldwide because of the improved short-term survival from myocardial infarction (MI) [73]. In the United States, it is estimated that the lifetime risk of developing HF is 1:5 [73]. Despite significant advances in the management of HF, 1-year mortality rates remain approximately 20%, with even higher

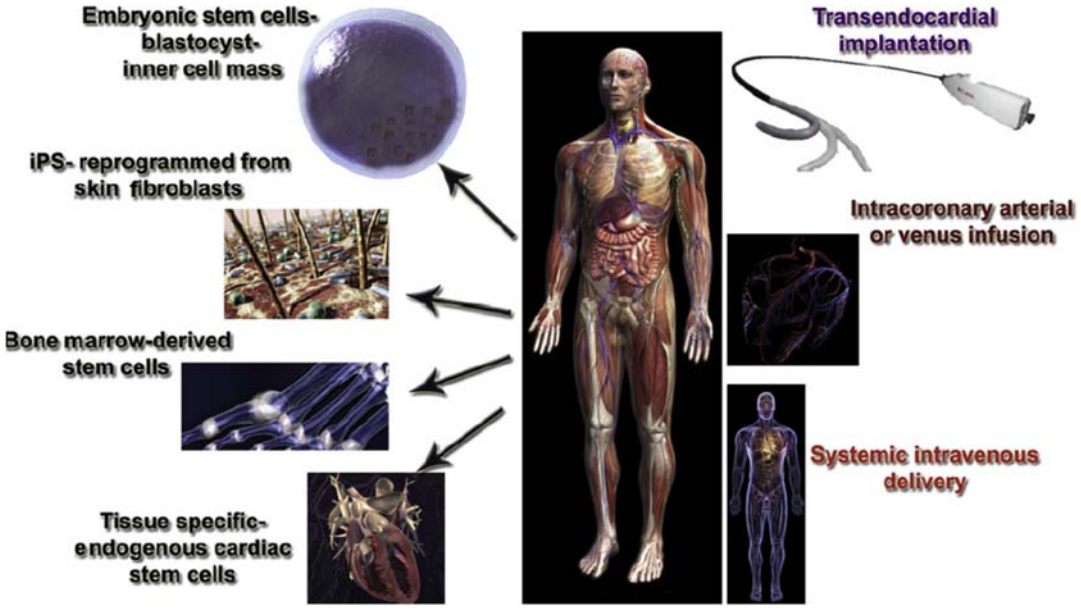


FIGURE 17.4 Stem cell–based therapeutic strategies for cardiac regeneration. Sources for stem cells with cardiac reparative capacities are numerous, including embryonic, bone marrow–derived, and cardiac-specific stem cells. After their expansion into therapeutic quantities, they can be transplanted into the patient using direct transendocardial implantation, intracoronary infusions, or subcutaneous intravenous delivery. *iPS*, induced pluripotent stem.

rates among patients hospitalized for HF [73]. Coronary heart disease is the predominant cause of HF in developed countries [73]. The pathophysiologic underpinning of this phenomenon is ventricular remodeling, which ensues after MI; therefore, regenerative therapies are targeted to preventing or reversing the remodeling process.

To date, most trials have employed BM-derived strategies to treat patients after acute myocardial infarction (AMI), with the goal of preventing remodeling largely through ameliorating infarct size [73]. Strategies have begun to be tested for revering remodeling in patients with HF and left ventricular (LV) dysfunction, both of ischemic and nonischemic etiology [75–80].

The basic premise underlying early attempts to repair cardiac injury is that of myocyte terminal differentiation [74]. Thus, early and ongoing attempts were designed around cell replacement with cells capable of differentiation. An early attempt at cell-based therapeutic myocardial replacement was reported in 1993, when Koh and coworkers used murine cardiomyocyte-like tumor cells to create intracardiac grafts [81]. By genetically manipulating murine hearts to overexpress oncogenes such as the simian virus 40 large T antigen, the investigators created a tumorigenic cardiomyocyte cell line with the capacity for proliferation both in vitro and in vivo. Implantation of these cells into healthy mouse hearts was accompanied by long-term engraftment in about 50% of the recipients without affecting heart function, introducing the notion of using cells to replace injured or lost cardiomyocytes. This experiment created a new field of investigation that has led to the exploration of multiple types and sources of cells as potential cardiac therapeutic agents. In 1998, Anversa and coworkers [82]reported evidence of mitosis in adult cardiomyocytes, which suggested cellular renewal throughout adult life. This discovery instigated the notion that myocyte renewal may not be attributed only to cardiomyocyte proliferation per se, but also to homing and differentiation of endogenous stem cells. To address this idea, Orlic and coworkers [83] demonstrated that transplantation of BM-derived, lineage-negative (Lin[−]/c-Kit[+]) stem cells into the infarcted mouse heart caused differentiation of the BM cells into cardiac myocytes and vessels and substantial recovery of cardiac function.

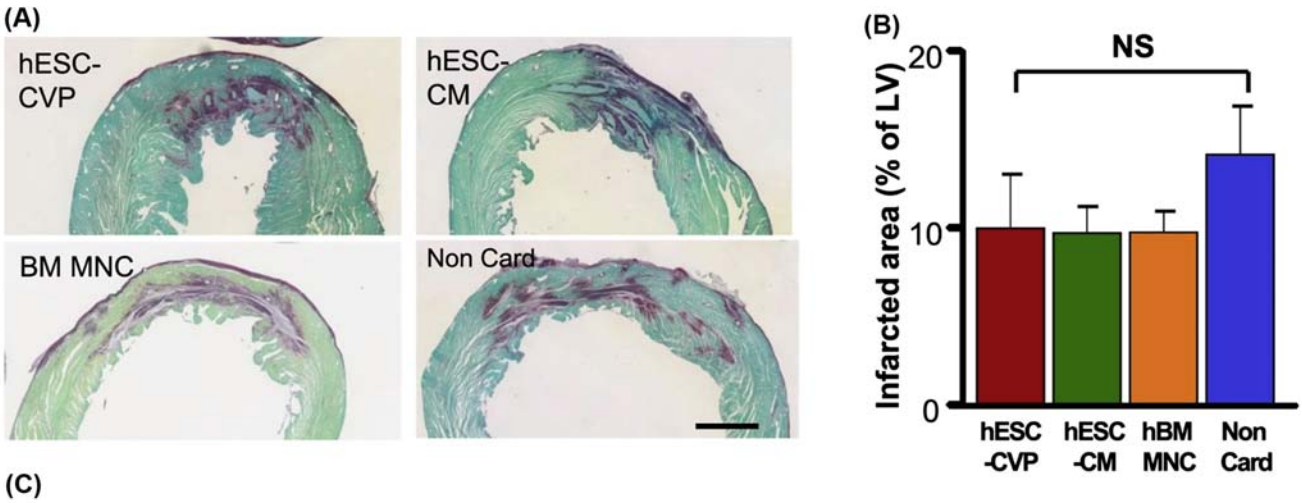
Cardiac engraftment of cells from distant sites was demonstrated in studies of sex-mismatched heart transplants [41]. In these studies, male cardiomyocytes were identified in female hearts transplanted into male patients (Fig. 17.1C and D). More important, the investigators also detected a subpopulation of cardiac precursor cells of donor and host origin, which suggested for the first time that the heart could contain its own CSC population. These observations opened up the field of adult stem cell–based therapy and led to a plethora of studies using multiple sources of cells to stimulate postinjury cardiac repair. Based on the observations by Orlic and colleagues, BM was preferentially used as a cell source, prompting a decade-long quest to establish the clinical value and mechanism of action of BM-derived, cell-based therapy for heart disease [73]. However, the quest is controversial and other

experimental observations have challenged the hypothesis of cell transdifferentiation as a dominant mechanism of action for cell-based cardiac repair [6,73,83,84].

MECHANISMS OF ACTION

Two major categories are thought to underlie the mechanisms of actions of cell-based therapeutics, each with varying degrees of experimental support: direct remuscularization and stimulation of endogenous repair (Fig. 17.5). The first category entails tissue-engineering approaches to remuscularize the dead, scarred myocardium by engrafting billions of new, healthy cardiac cells manufactured in the laboratory from pluripotent stem cells [85–87] or via genetic conversion of adult fibroblasts [88,89].

The second category entails using adult stem cells, isolated and expanded in vitro from the heart or extracardiac tissues and infused through the bloodstream or engrafted endomyocardially to stimulate endogenous mechanisms of heart regeneration. Most transplanted adult stem cells do not become heart muscle cells themselves, but induce



(C)

OUTCOME	PSC-Based Strategies for Direct Remuscularization	Endogenous Repair by Adult Stem Cells
ENGRAFTMENT	↑	↓
REMUSCULARIZATION	↑	Limited*
ENDOGENOUS CARDIOMYOCYTE TURNOVER	?	↑
NEOVASCULARIZATION	?	↑
SCAR SIZE REDUCTION	–	↑
HEART FUNCTION	–	↑
ANATOMICAL IMPROVEMENTS	–	↑
ARRHYTHMIAS	↑	–

FIGURE 17.5 Cell-based therapeutic strategies for cardiac repair. (A, B) The two major categories of cell therapy are direct remuscularization strategies with human embryonic stem cell (hESC) or induced pluripotent stem cell (iPSC)-derived cardiomyocytes (CM) and progenitors (CVP), or stimulation or endogenous repair with adult stem cells (i.e., bone marrow mononuclear cells (BM MNC). Both strategies exert similar effects in cardiac repair. (C) Comparison of the mechanisms of action underlying the two therapeutic strategies. *Differentiation of adult stem cells (i.e., MSCs) to cardiomyocytes is minimal but not absent. *Non Card*, noncardiac. *(A, B) Adapted from Fernandes S, Chong JJ, Paige SL, Iwata M, Torok-Storb B, Keller G, Reinecke H, Murry CE. Comparison of human embryonic stem cell-derived cardiomyocytes, cardiovascular progenitors, and bone marrow mononuclear cells for cardiac repair. Stem Cell Rep 2015;5:753–62. https://doi.org/10.1016/j.stemcr.2015.09.011; (C) Hatzistergos KE, Hare JM. Cell therapy: targeting endogenous repair versus remuscularization. Circ Res 2015;117;659–61. https://doi.org/10.1161/CIRCRESAHA.115.307346.*

cardiac repair through other mechanisms, including stimulation of myocardial regeneration from endogenous cardiac progenitors and cardiomyocyte mitosis [10,90,91], fusion with host cells to promote survival and induce regenerative activity [6,49,92], as well as secretion of paracrine factors and exosomes that exert antiinflammatory, antifibrotic, and angiogenic effects [10,75,93–97].

CLINICAL TRIALS

The field of stem cell research has gained enormous attention. Experimental work has employed both pluripotent stem cell–derived and tissue-specific adult stem cells to treat heart disease [73]. Whereas consensus has emerged that there is some functional merit to cell-based therapies for heart disease [73,98–100], underlying mechanisms of action remain controversial. In addition to this central issue, other key issues that need to be settled include the role of host factors in cell functionality [101] and the exciting possibility of using allogeneic grafts because of the unique immunoprivileged properties of some cell types [75]. Despite much controversy, substantial work in the clinical arena with trials of growing size and sophistication has produced a major database of safety and efficacy that is paving the way to future clinical development. We next review developments using each cell-based strategy.

Cardiopoietic Stem Cells

Pluripotent Stem Cells

ESCs, the prototypic pluripotent stem cell, were first isolated from mice in 1981 [102,103] and subsequently from human blastocysts in 1998 [104]. These cells arise from the inner cell mass of late blastocysts and differentiate into cell types of all three germ layers, including cardiomyocytes. From a clinical standpoint, because undifferentiated ESCs are pluripotent, they have a high oncologic risk when they are used as a cell-based therapeutic. However, differentiation of ESCs to cardiac progenitors or cardiomyocytes before transplantation overcomes this important limitation and reduces the risk of tumor formation [105,106]. Early animal studies suggested that ESC-derived, cardiac-committed cells may have the capacity to improve myocardial function and structure after MI by directly remuscularizing the infarct area with new cardiomyocytes and vessels. However, later experiments revealed that the degree to which engraftment of human ESC-derived cardiac cells reduces scar size and improves heart function in animals with experimental myocardial ischemia is at best comparable to that seen with adult cell-based therapy (Fig. 17.5) [73,74,85–87,107]. Furthermore, although engraftment of human ESC-derived beating cardiomyocytes into the myocardial scar appears to be safe from an oncologic standpoint, it has been associated with an increased risk for arrhythmogenesis, which suggests that more work is necessary to improve the safety of this approach before clinical testing [85]. To date, the Transplantation of Human Embryonic Stem Cell-derived Progenitors in Severe Heart Failure trial (NCT02057900) is the only ESC-based clinical trial in the field of cardiovascular diseases to test the safety and feasibility of transplanting human ESC-derived cardiac progenitor cells expressing Isl1 and SSEA1 in patients with severe HF [108].

Another strategy to obtain pluripotent stem cells involves the induction of pluripotency in somatic cells through genetic reprogramming, so-called iPSCs. In a pioneering study conducted by the laboratory of Shinya Yamanaka [109,110], adult mouse and human skin fibroblasts were induced into pluripotent embryonic-like iPSCs by transfection with stem cell–related genes such as *Lin28*, *c-myc*, *oct 4*, *sox 2*, *klf-4*, and *Nanog*. This approach has stimulated enormous enthusiasm given the potential to develop pluripotent stem cells without using human embryos and offers substantial availability of the cells. Not the least advantage of this approach is the prospect of developing host-tailored stem cells that could escape immune rejection, or the development of pluripotent cell lines from hosts with genetic diseases providing an optimal in vitro experimental system for disease-modeling and drug-screening applications [111]. However, the production and derivation of patient-specific, autologous heart cell grafts from iPSCs still require expensive and labor-intensive processes, which makes the clinical translation of personalized, iPSC-based regenerative medicine practically unfeasible. An alternative strategy proposed is the development of immunocompatible iPSC master cell banks that can be used to derive "off-the-shelf" allogeneic grafts. Nonetheless, preclinical animal models of heart regeneration using immunocompatible, allogeneic, iPSC-based strategies indicate that engraftment of iPSC-derived cardiac cells still requires high-dose immunosuppression. In addition, similar to ESC-based strategies, direct remuscularization from iPSC-derived cardiac cells is disappointingly inefficient and may have important adverse effects, including arrhythmogenesis (Fig. 17.6) [112]. Other studies indicate that, similar to adult cell-based therapeutics, iPSC-based therapeutics may

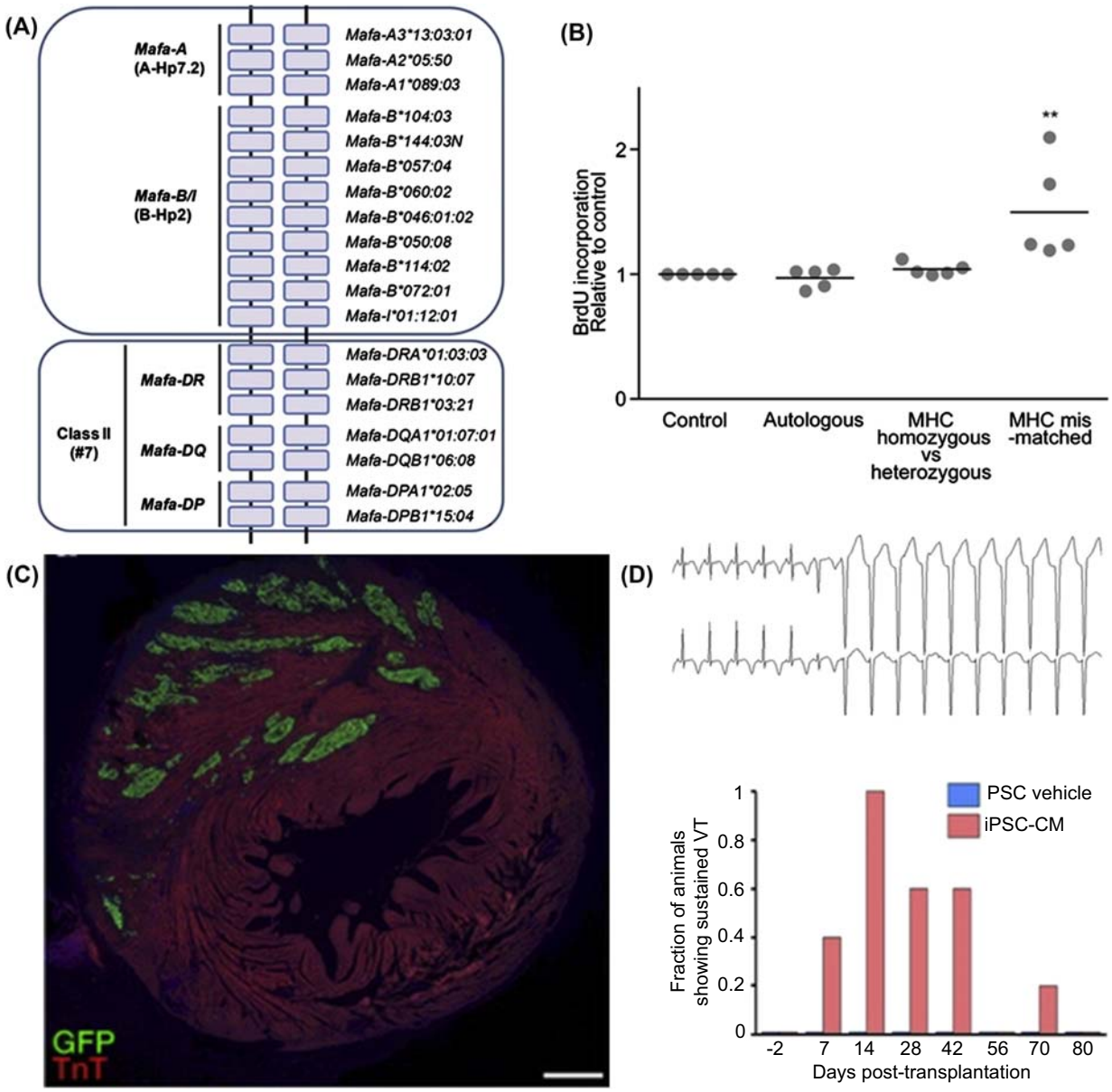


FIGURE 17.6 Induced pluripotent stem cell (iPSC)-based strategies for cardiac repair. A proof-of-concept preclinical study tested the allogeneic transplantation of beating cardiomyocytes derived from major histocompatibility complex (MHC) homozygous monkey iPSCs (A) into MHC heterozygous recipients. The allogeneic cardiomyocytes were not immunogenic in vitro (B) but required high-dose immunosuppression for in vivo engraftment (C). Another major concern raised by the study is that engraftment of terminally differentiated, allogeneic cardiomyocytes significantly increased the risk for arrhythmogenesis, whereas the effects on heart function and scar size were far from optimal (D). Future allogeneic iPSC-based strategies should focus on developing novel, cost-effective bioengineering approaches to enhance immunotolerance, improve engraftment, and reduce arrhythmogenicity of the allografts. *BrdU*, bromodeoxyuridine. *Adapted from Shiba Y, Gomibuchi T, Seto T, Wada Y, Ichimura H, Tanaka Y, Ogasawara T, Okada K, Shiba N, Sakamoto K, et al. Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. Nature 2016;538:388–91. https://doi.org/10.1038/nature19815. Epub 2016 Oct 10.*

be useful for bioengineering cardiovascular patches to stimulate the heart's innate repair mechanisms [96]. To date, iPSC cells have not entered the clinic for heart-related diseases.

Adult Stem Cells

The field of CSC therapy has been advanced substantially through the discovery that adult stem cells have the capacity to (trans)differentiate into lineages other than their tissue of origin. This stem cell plasticity allows the use of stem cells isolated from a variety of easily accessible sources such as BM, peripheral blood, fat, umbilical

cord, skeletal muscle, and the heart itself to be used for cell-based cardiac repair. The first generation of cell-based clinical trials was conducted with cells of extracardiac tissue origin and showed MSCs to be the safest and most promising strategy for stimulating the innate regenerative response mechanisms of the failing human heart [73,98]. The second generation of cell-based therapy supported the concept that the adult human heart contains its own stem cell compartment that may be expanded in culture and delivered back to the damaged heart muscle to enhance cardiac repair. Finally, given the stimulatory effects of MSCs on tissue-specific CSCs (Fig. 17.3), the third generation of cell-based clinical trials is testing whether the combined engraftment of the two stem cell types yields an additive regenerative effect [73,80,113].

Bone Marrow Stem Cells

Because of its various well-defined stem cell compartments and its ease of access, whole-BM and BM-derived mononuclear stem cells (BMMNCs) are the most widely studied type of cell for cellular cardiomyoplasty. Using different cell-surface markers, BMMNCs can be fractionated to hematopoietic stem cells (HSCs) or nonhematopoietic stem cells. The latter includes a number of distinct subtypes named SPs [114], endothelial progenitor cells (EPCs) [115], MSCs [73], multipotent adult progenitor cells [116], multilineage inducible cells [117], and very small embryonic-like stem cells [118].

Bone Marrow–Derived Mononuclear Stem Cells

Numerous experimental and clinical studies have tested BMMNCs for a range of therapeutic strategies involving their transplantation and/or mobilization to sites of cardiac injury. The totality of evidence of trials of BM cells and derivatives supports both the safety and provisional efficacy of this approach. Three metaanalyses evaluating data from approximately 18 trials and close to 1000 patients [7,100,119] concluded that BM cell–based therapies contribute to modest improvements in cardiac function by reducing infarct size, preserving LV dimensions, and increasing ejection fraction by 2%–3% within 6 months after transplantation. Long-term follow-up data derived from the Brain Oxygen Optimization in Severe Traumatic Brain Injury and Outcomes for Antiretroviral Therapy Patients Receiving Palliative Care (REPAIR)-AMI studies documented that the therapeutic results are sustained up to 5 years posttransplantation [73]. One of the most exciting observations derives from the Data and Safety Monitoring Board data of the REPAIR-AMI study [119a]. In this study, 204 patients with AMI underwent successful reperfusion of the culprit coronary vessel(s), and 3–7 days later were randomized to receive intracoronary infusion of autologous BMMNCs or placebo. By 4 months, patients who had received the cells showed a significantly improved left ventricular ejection fraction (LVEF) compared with the placebo, and the ones with larger infarcts (baseline LVEF $< 48.9\%$) were more responsive to the therapy than the others. Importantly, 1-year follow-up data from REPAIR-AMI demonstrated improved event-free survival (death, recurrence of MI, revascularization, or rehospitalization for HF) of the BMMNC-treated patients compared with the placebo. In addition to the post-MI setting, several trials employed BMMNCs for patients with established LV dysfunction and/or HF owing to either ischemic or non-=ischemic causes; data are sufficiently promising to warrant further study [73].

Endothelial Progenitor Cells

This subset of hematopoietic stem cells can be isolated from BMMNCs as well as peripheral blood mononuclear cells (PBMCs), based on the expression of HSC surface markers such as CD34, CD133, and vascular endothelial growth factor receptor 2. The main mechanism of action of these cells is the formation of new vessels in the infarcted myocardium; however, little evidence exists for their in vivo transdifferentiation into new cardiac myocytes [73]. In rats with AMI, intravenously injected EPC stimulated the development of collateral vessels from preexisting vessels as well as de novo capillary formation [120]. This was associated with decreased apoptosis of myocytes in the borderline zone, reduced fibrosis and scar formation resulting in prevention of LV remodeling, and improvement in myocardial function [120]. Infusion of EPC in the infarct-related arteries can improve vasomotor function, an effect that could contribute to improved myocardial function [121]. In patients with old MI and chronic coronary total occlusion, intracoronary infusion of EPC after recanalization of the occluded artery improved myocardial perfusion, reduced infarct size, and ameliorated myocardial function [122].

However, a clinical trial that compared the effects of granulocyte colony-stimulating factor and PBMCs as an alternative approach to recruit EPCs at the sites of MI was terminated prematurely owing to the potential adverse reaction of increased restenosis [123]. Approaches that involve EPC therapies combined with gene therapies or even the genetic manipulation of EPCs before transplantation have emerged in an attempt to minimize side effects and improve outcomes (NCT00936819).

Mesenchymal Stem Cells

The MSC, an adult stem cell with self-replication and multilineage differentiation capacity, represents a promising adult stem cell for regenerative medicine. MSCs can be isolated from a variety of tissues such as heart, adipose, umbilical cord/umbilical cord blood, and BM, although whether they all share common cardiopoietic and immunomodulatory properties remains unclear [73]. MSCs lack hematopoietic lineage markers such as CD14, CD34, and CD45 and express specific stromal cell-surface markers such as Stro1, CD105, CD90, and CD71. They adhere to plastic surfaces and grow as cell monolayers without losing their stem cell phenotype. Furthermore, they have reduced expression levels of major histocompatibility complex (MHC) class-I molecule and lack MHC class-II (although interferon-gamma will induce MHC class-II) and costimulatory molecules CD80 (B7-1), CD86 (B7-2), and CD40. MSCs are therefore the prototypic immunoprivileged cell-based therapy and have been tested in phase I double-blind, randomized clinical trials as an allogeneic graft [75,76,95,124,125].

The mechanism of action of MSCs as a cardiac regenerative agent appears to be multifactorial. Although definite evidence is reported of their in vivo transdifferentiation into cardiovascular elements, the degree to which they differentiate does not fully explain their substantial cardiac reparative properties [90,126,127]. Indeed, MSCs appear to have additional powerful effects mediated by secreted factors and cytokines that evoke the therapeutic response [10,75,94,95]. However, data document that MSCs facilitate cardiac regeneration through mechanisms that involve both differentiation and paracrine stimulation of innate repair pathways [10,90,128]. In particular, MSCs seem to have a unique capacity to gain control over the endogenous c-Kit$^+$ cardiac precursor cell content and establish the necessary cardiopoietic cues that instruct the latter to regenerate a myocardial scar [10,80,90,92,113,129].

In various animal models of AMI, intramyocardial injection of MSCs prevents ventricular remodeling by reducing scar formation, leading to a net improvement in myocardial function (Fig. 17.7) [126,127]. However, some studies in mice with AMI failed to show a sustained benefit of MSC therapy despite an early benefit [73]. In patients with AMI, intracoronary MSC infusion improved myocardial perfusion and function and reduced LV end-systolic and end-diastolic volumes [130]. In another small study, combined intracoronary administration of MSC and EPC improved perfusion and contractility of the infarcted area [131]. Although early uncontrolled animal studies suggested that intracoronary injection of MSCs could induce coronary artery occlusion and MI [132], this was not observed in humans [130]. A 53-patient, double-blind, placebo-controlled, phase I clinical study of allogeneic MSCs administered intravenously within 10 days after AMI demonstrated acute and long-term safety of the approach [124]. Although this study was primarily designed to test safety, four domains of prespecified safety monitoring supported an improved outcome in the patients treated with cells. These included a reduction in malignant ventricular arrhythmias, improved pulmonary function, an improved EF in the subset of patients with anterior MI, and finally, an improved patient well-being score at 6 months [124]. These provocative data led to the conduct of a completed phase II study, the results of which will be reported (NCT00877903). Similarly, in the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (NCT01216995) and Allogeneic Mesenchymal Precursor Cell Infusion in Myocardial Infarction (NCT01781390) trials, patients with AMI were treated with adipose tissue−derived or STRO3-selected BM MSCs, respectively, via the intracoronary route. A third clinical study in which patients with AMI are treated with STRO3-selected MSCs via intramyocardial injection is also under way (NCT00555828).

The safety and efficacy of MSC-based therapeutics has also been demonstrated in patients with chronic ischemic heart disease. The Prevention of Contrast Renal Injury With Different Hydration Strategies [76] and Transendocardial Injection of Autologous Human Cells in Chronic Ischemic Left Ventricular Dysfunction and Heart Failure Secondary to Myocardial Infarction [77] trials compared autologous BM MSCs versus allogeneic MSCs and BMMNCs respectively, delivered transendocardially in patients with chronic ischemic cardiomyopathies. Both studies demonstrated that at 1 year, treatment with MSCs, but not BMMNCs, resulted in scar size reductions and improved contractility and functional capacity and reversal of LV remodeling (Fig. 17.8). Similarly, a phase I/II, randomized, double-blinded, placebo-controlled study of the safety and efficacy of intramyocardial injection of autologous MSCs in patients with chronic ischemic LV dysfunction resulting from MI undergoing cardiac surgery for coronary artery bypass grafting (the Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery trial) found that MSC injections into akinetic yet nonrevascularized segments produces comprehensive regional functional restitution compared with revascularized and non−MSC treated segments, which in turn drives improvement in global LV function [78].

In a rat model of nonischemic dilated cardiomyopathy, intramyocardial injection of MSC exerted antifibrotic effects and improved myocardial perfusion and function [133]. Similarly, a randomized study comparing allogeneic versus autologous MSCs in 37 patients with nonischemic dilated cardiomyopathy (Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis in Dilated Cardiomyopathy Trial, NCT01392625) revealed that intramyocardial injection of MSCs was highly safe and efficacious [75]. Strikingly, this study demonstrated that patients receiving

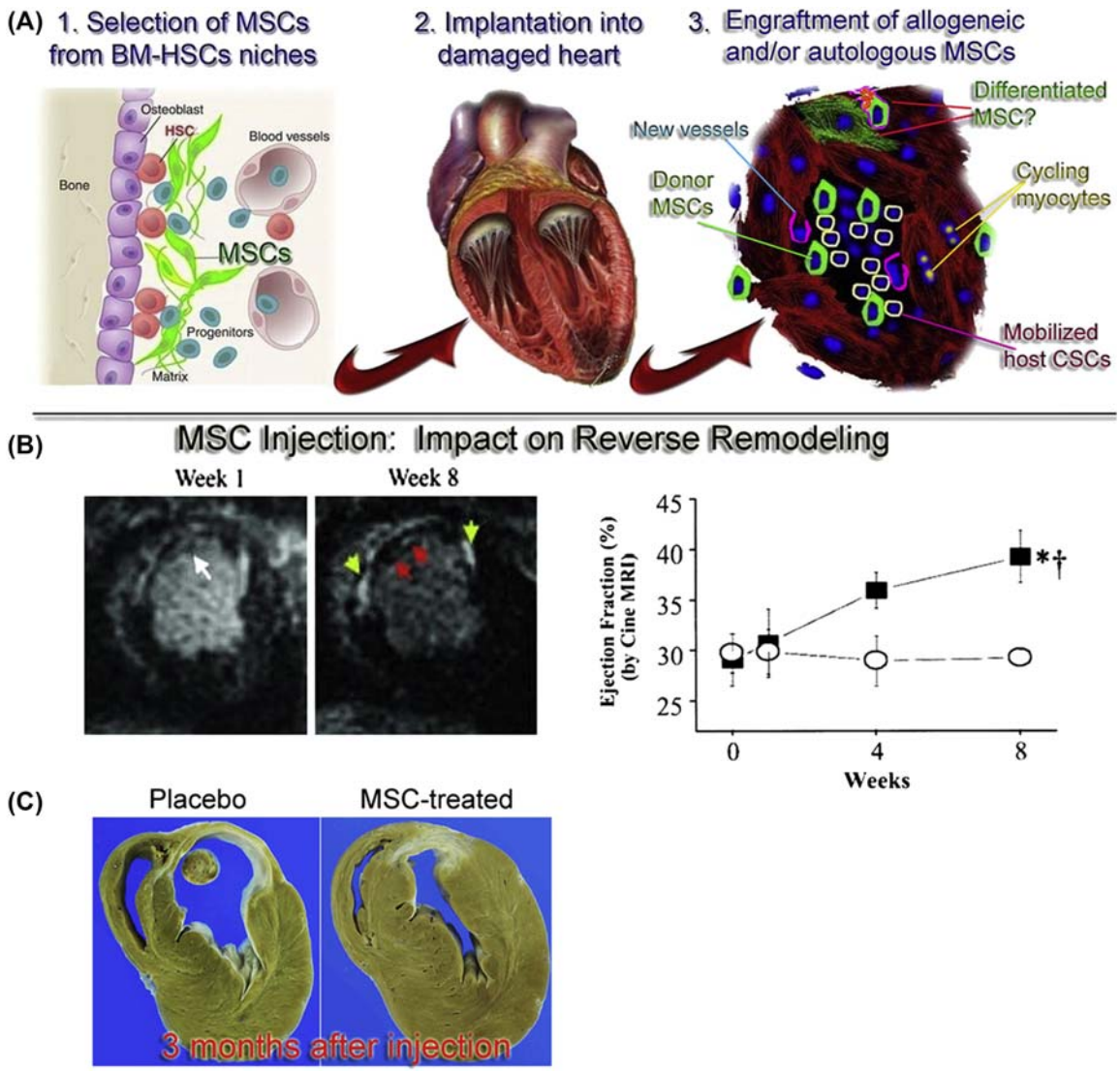


FIGURE 17.7 (A) Mesenchymal stem cells (MSCs) can provide a safe allogeneic source for cell-based therapies. Their mechanisms of action are believed to be multifaceted because activation of innate (stimulation of angiogenesis, cardiomyocyte proliferation, and cardiac stem cell [CSC] mobilization) and exogenous (differentiation onto cardiovascular lineages) repair pathways have been reported. (B) Prevention of remodeling in the porcine heart. Cardiac magnetic resonance imaging and multidetector computed tomography document the development of a subendocardial rim after MSC transplantation in the damaged zones of infarcted hearts. The newly formed tissue rendered an approximately 50% decrease in infarct size and restoration of the heart function. (C) Reverse remodeling induced by MSC injection in the porcine heart. *BM-HSCs*, bone marrow hematopoietic stem cells.

allogeneic therapy benefited more than did those receiving autologous cells, as indicated by significant differences in EF, 6-min walk test, Minnesota Living with Heart Failure Questionnaire, endothelial function, and, most important, a dramatic remodeling of immune cells toward a less inflammatory/exhausted phenotype [75] (Fig. 17.9). These findings are on par with earlier reports indicating that EPCs and BMMNCs from patients with established coronary heart disease or cardiovascular risk factors show impaired proliferating and migratory capacity [134,135]. Therefore, treatment of patients with cardiovascular diseases with MSCs obtained from healthy donors may be equally safe as and more advantageous than using autologous MSCs.

The total database of studies of MSC therapy for ischemic and nonischemic cardiomyopathies includes several proof-of-concept clinical trials described previously. Together, these studies illustrate that MSCs can be used successfully as either autologous or allogeneic grafts to treat heart disease [73]. Although safety has served as the primary end point thus far, an overall provisional efficacy of MSCs on reversing heart disease has also been strongly suggested. All studies report significant improvements in cardiac function accompanied by a substantial reduction in scar size. As a result, MSCs are now in phase III trials for AMI and chronic MI. In a phase I Cardiopoietic Stem Cell

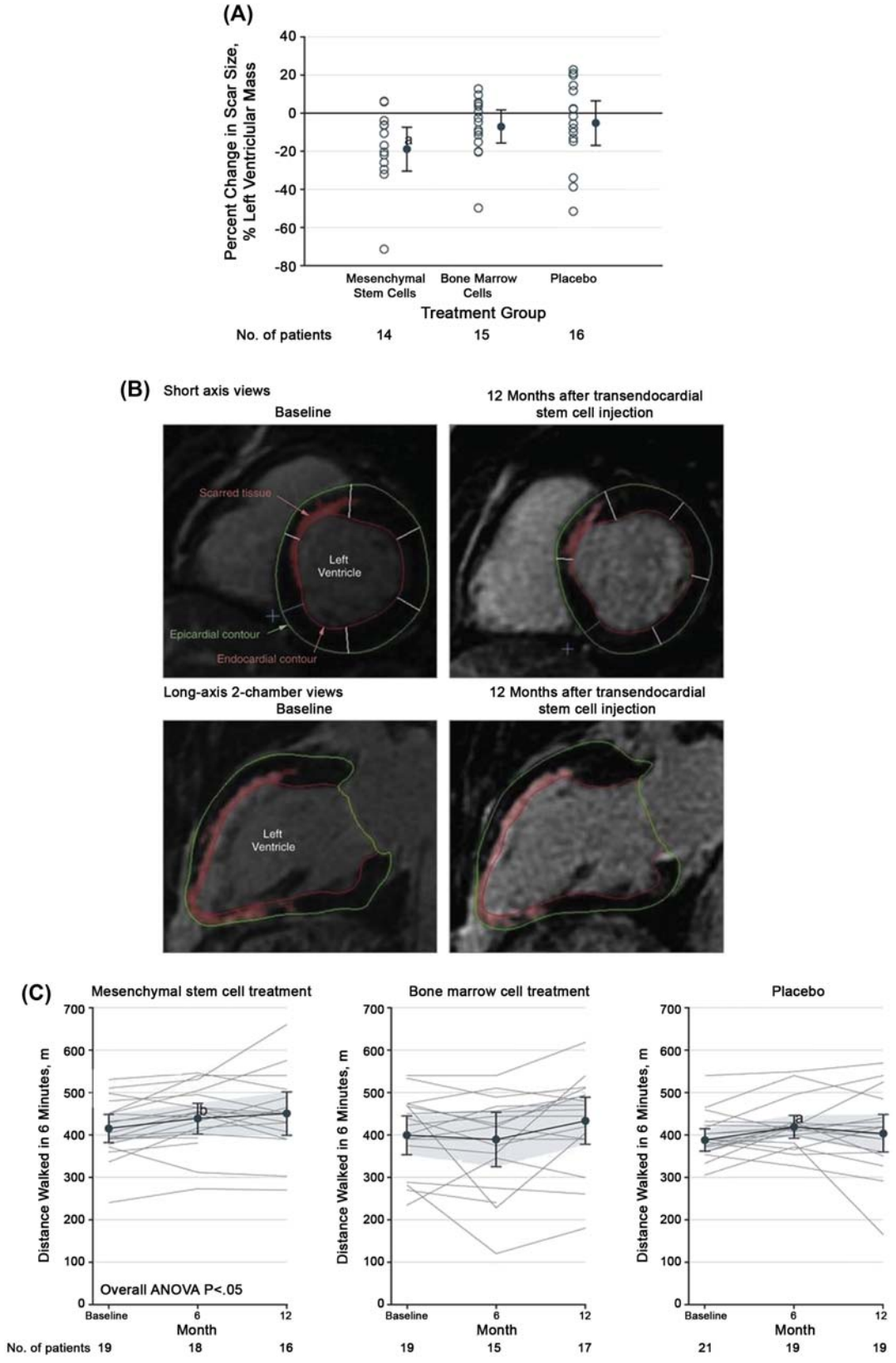


FIGURE 17.8 Transendocardial Injection of Autologous Human Cells (bone marrow or mesenchymal) in Chronic Ischemic Left Ventricular Dysfunction and Heart Failure Secondary to Myocardial Infarction trial: percent change in scar size as a percentage of left ventricular mass and impact on functional capacity. (A) The 14 patients treated with mesenchymal stem cells exhibited a significant reduction in scar size ($P = .004$) as a

Therapy in Heart Failure trial, patients with chronic ischemic cardiomyopathy who received intramyocardial injection of autologous BM MSCs pretreated with a cardiogenic cocktail reported significant improvements in LVEF, left ventricular end-diastolic volume, functional capacity, and quality of life compared with those receiving the standard of care [136]. This strategy was not borne out in the phase III Congestive Heart Failure Cardiopoietic Regenerative Therapy study, which used a composite end point (all-cause mortality, worsening HF, Minnesota Living with Heart Failure Questionnaire score, 6-min walk distance, LV end-systolic volume, and ejection fraction) to evaluate the safety and efficacy of autologous MSCs pretreated with the cardiopoietic cocktail to treat ischemic heart failure. Although the procedure was safe, efficacy between groups at 39 weeks posttreatment (the primary outcome measure) was neutral [137].

Myoblasts

Skeletal myoblasts were the first contractile cell type transplanted in the infarcted heart with the goal of restoring cardiac function [138]. These cells can be purified from the skeletal muscle of the patient; after expansion into therapeutic quantities, they can be transplanted into the myocardium. Transplantation of these cells to the heart is accomplished either surgically or by a catheter delivery system [139]. There have been several well-conducted phase I/II clinical studies [140–142]. The Medical Research Council Adjuvant Gastric Infusional Chemotherapy trial revealed that there was a dose-dependent attenuation in LV remodeling, but that it was not accompanied by functional improvements in cardiac function (Fig. 17.10). In addition, compared with MSCs, there are concerns that skeletal myoblast can precipitate arrhythmias (Fig. 17.10C) [140,142].

In experimental models of AMI, intramyocardial injection of myoblasts preserves myocardial function and abrogates the remodeling process [143]. Myoblasts can differentiate into slow-twitch myotubes in the infarcted area, which can contribute to myocardial systole [143]. It has been suggested that skeletal muscle–derived stem cells have greater potential for myocyte regeneration than myoblasts and can also stimulate innate angiogenesis. This cell population appears to be more effective in improving myocardial perfusion and contractility and attenuating remodeling in animal models of MI [144]. The failure of myoblasts to improve cardiac function in humans has been attributed to their inability to differentiate into cardiac myocytes and the in situ development of dysfunctional electrical coupling with resident cardiomyocytes (Fig. 17.10) [145]. Accordingly, some studies are focused on identifying and characterizing a more cardiogenic skeletal muscle-derived cell population that may improve cardiac repair [146].

However, studies have indicated that transplantation of skeletal myoblasts, bioengineered ex vivo into cell sheets, in patients with HF caused by ischemic heart disease is safe, nonarrhythmogenic, and clinically advantageous in facilitating LV reverse remodeling, improving HF symptoms, and preventing cardiac death, compared with cardiac resynchronization therapy [141]. Based on these studies, the Japanese government issued a 5-year conditional approval of autologous skeletal myoblast sheet transplantation as the first cell-based regenerative medicine product worldwide for use in patients with severe HF (EF $< 35\%$; III or IV on the New York Heart Association functional classification scale) [147].

Cardiac Stem Cells

CSCs are tissue-specific stem cells that reside within the heart itself. Cardiac progenitors were first reported in 2002, when Hierlihy et al. detected robust SP cells in the postnatal murine heart that expressed the adenosine triphosphate–binding cassette transporter Abcg2 and extruded Hoechst dye [148]. These cells represented about 1% of total cardiac cells and differentiated into cardiac myocytes in vitro. After this observation, in 2003, two different groups, Beltrami et al. [46] and Oh et al. [64], reported the isolation and characterization of two novel CSCs from the murine heart. These resident stem cells have been reported to correspond to 0.01%–2% (or approximately 1 CSC for every 13,000 cardiomyocytes) of the total cell population of the human heart and are mostly

percentage of left ventricular mass; there were no differences for the 15 patients treated with bone marrow cells or the 16 patients in the placebo group. (B) Short-axis and long-axis views of the basal area of a patient's heart, with delayed tissue enhancement delineated at the septal wall (short axis) and anterior and inferior walls (long axis) as well as the entire apex (long axis). Delayed tissue enhancement corresponds to scarred tissue and is depicted as brighter than the nonscarred tissue. The *red, green, and white lines* demarcate the endocardial and epicardial contours and borders of the segments, respectively. Twelve months after injection of mesenchymal stem cells, the scar mass was reduced from 30.85 g at baseline to 21.17 g (short axis) and delayed tissue enhancement receded in the midinferior and basal anterior walls (long axis). (C) Patients in the mesenchymal stem cell group exhibited a significant increase in 6-min walk distance when 6- and 12-mo time points were compared with baseline in a repeated-measures model ($P = .03$). No significant difference was observed for patients in the bone marrow cell group ($P = .73$) or the placebo group ($P = .25$). *ANOVA*, analysis of variance. *Adapted from Golpanian S, Wolf A, Hatzistergos KE, Hare JM. Rebuilding the damaged heart: mesenchymal stem cells, cell-based therapy, and engineered heart tissue. Physiol Rev 2016;96:1127–68. https://doi.org/10.1152/physrev.00019.2015.*

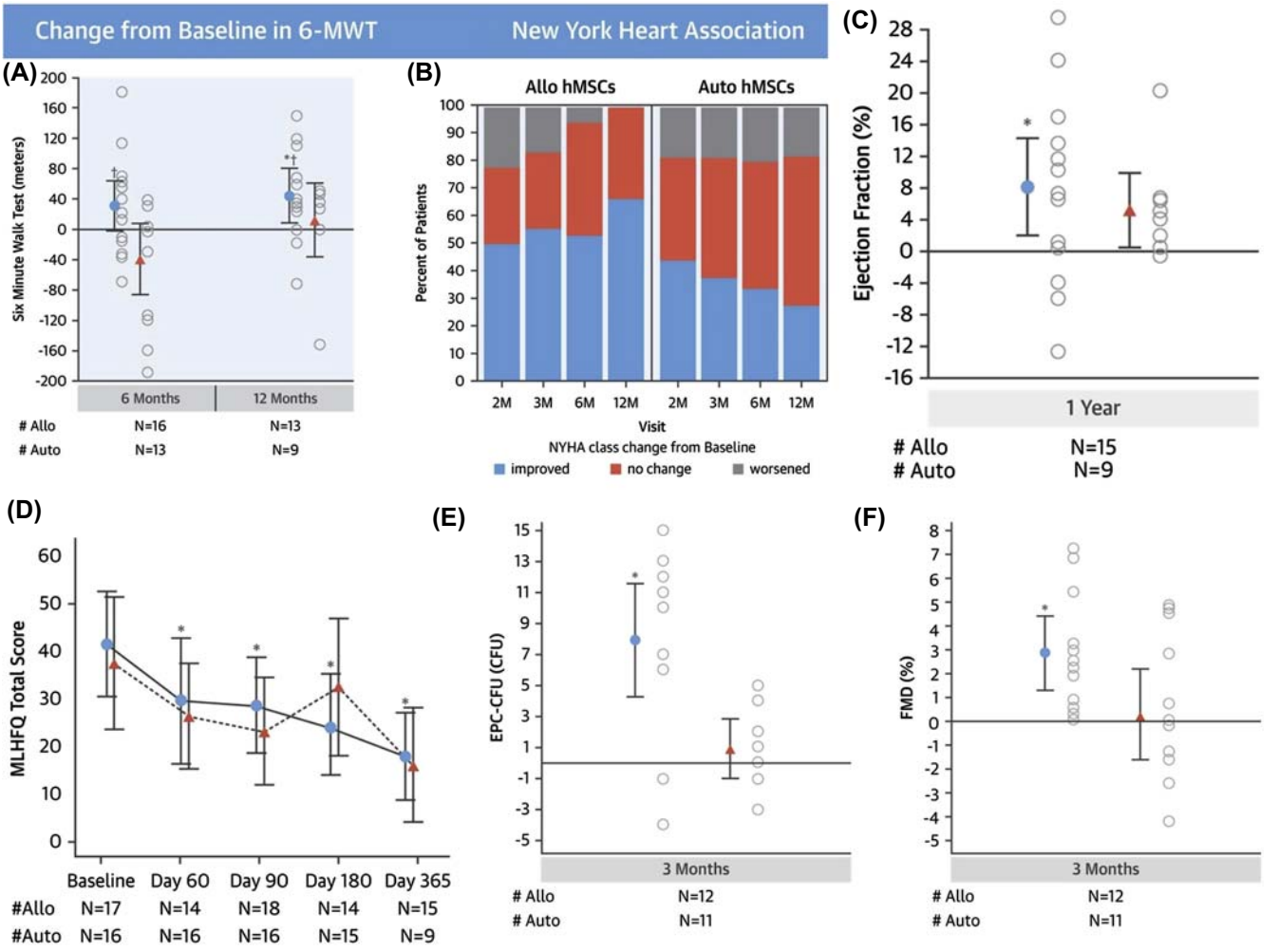


FIGURE 17.9 **Cell therapy with allogeneic mesenchymal stem cells (MSCs) may be more beneficial than autologous MSCs in patients with dilated cardiomyopathy of nonischemic etiology (NIDCM).** One-year results from a randomized comparison of allogeneic versus autologous MSC therapy in patients with NIDCM (Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis in Dilated Cardiomyopathy Trial) support that treatment of heart failure with MSCs may be further improved if the cells are of a healthy allogeneic rather than autologous origin. More specifically, treatment with allogeneic MSCs (Allo hMSCs) from young, healthy donors, results in greater improvements in 6-min walk test (6-MWT) (A), New York Heart Association class change (B), ejection fraction (C), and Minnesota Living with Heart Failure Questionnaire scores (MLHFQ) (D) compared with autologous MSCs. In addition, endothelial function is significantly improved, as indicated by the amount of endothelial progenitor colony-forming units (EPC-CFUs) from peripheral blood samples (E) and percent changes in flow-mediated vasodilation (FMD) brachial artery diameter (F). *Solid blue circle,* Allo hMSC; *solid red triangle,* Auto hMSC. *Adapted from Hare JM, DiFede DL, Castellanos AM, Florea V, Landin AM, El-Khorazaty J, Khan A, Mushtaq M, Lowery MH, Byrnes JJ, et al. Randomized comparison of allogeneic Vs. Autologous mesenchymal stem cells for non-ischemic dilated cardiomyopathy: POSEIDON-DCM trial. J Am Coll Cardiol November 14, 2016.pii: S0735-1097(16)36906-6.*

recognized according to the expression of three cell-surface markers: c-Kit (the receptor for stem cell factor), multidrug resistance protein-1 (MDR-1), and/or Sca-1. CSCs are self-renewing, clonogenic, and multipotent and are able to differentiate both in vitro and in vivo into myogenic, endothelial, and vascular smooth muscle lineages. Two different methods for isolating human CSCs have been reported, but whether the purified cells share the same properties is yet unknown. The first method involves homogenizing relatively large amounts of cardiac tissue (about 30–60 mg for successful isolation) and subsequent antibody-based selection of CSCs [45]. The applicability of this method is limited to patients who undergo major cardiac interventions such as coronary artery bypass grafting, left ventricular assist device placement, or heart transplantation. The second method has been adapted from the field of neurological sciences and involves the culture of a single biopsy from which CSCs are selected with antibodies as a subpopulation of the outgrowing cells. Alternatively, CSCs can be selected without using antibodies, based on their sphere-forming property [149]. The discovery of adult CSCs represents a major biological discovery furthering understanding of cardiac pathophysiology and facilitating cardiac cell–based therapeutics.

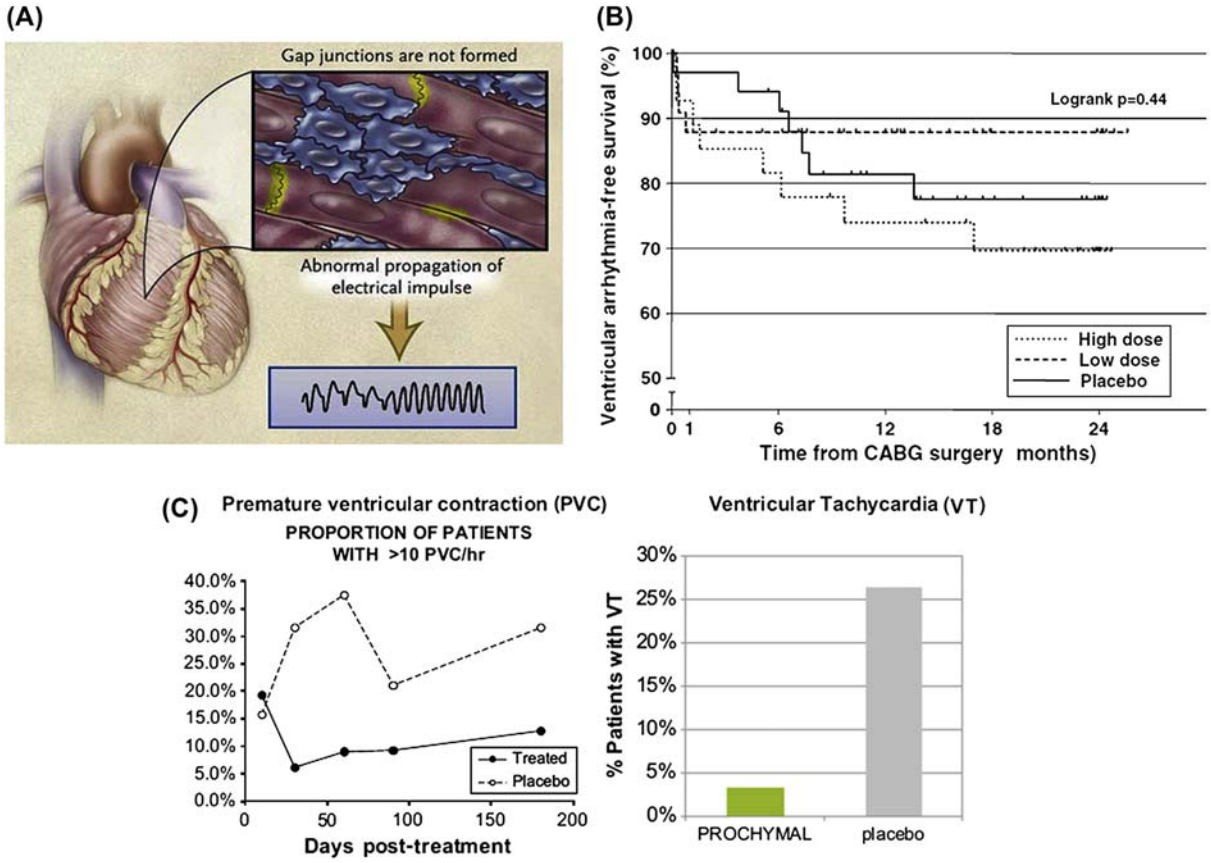


FIGURE 17.10 (A) Experimental studies have shown that skeletal fibroblasts engraft and survive in the damaged myocardium. However, these contractile cells do not express gap junctional proteins such as connexin-43 and fail to couple with the host myocytes. As a result, the transplanted cells cannot propagate the conduction signals, resulting in an arrhythmogenic substrate. (B) In the Medical Research Council Adjuvant Gastric Infusional Chemotherapy trial, patients with ischemic cardiomyopathy who received skeletal myoblasts were more prone to developing arrhythmias compared with the placebo-treated group. (C) Mesenchymal stem cell, in contrast, have an antiarrhythmic effect. *CABG*, coronary artery bypass grafting.

c-Kit$^+$ Cardiac Stem Cells

c-Kit$^+$ CSCs represent a promising candidate for cardiac-specific stem cell lineages. This cell type is described in multiple species ranging from rodents to large animals to humans and is likely heterogenous in nature [15]. Endogenous cardiac repair mechanisms are thought to involve the mobilization of c-Kit$^+$ CSCs to areas of cardiac injury soon after infarction [90,150]. However, genetic lineage fate-mapping experiments in mice suggested that endogenous cardiomyocyte regeneration from adult c-Kit$^+$ CSCs is likely minimal [49]. On the other hand, animal studies support that implantation of culture-expanded c-Kit$^+$ CSCs, genetically modified or not, can reduce infarct size and improve cardiac function, largely through paracrine effects [151,152] (Fig. 17.11). These findings led to the Cardiac Stem Cells in Patients With Ischemic Cardiomyopathy (SCIPIO) trial, the first phase I clinical trial of c-Kit$^+$ CSCs in patients with ischemic heart disease (www.clinicaltrials.gov, NCT00474461). The 1-year follow-up results from the SCIPIO trial support that intracoronary infusion of one million autologous c-Kit$^+$ CSCs in patients with HF of ischemic etiology undergoing coronary artery bypass grafting is safe and feasible and may promote significant improvements in global and regional LV function, decrease scar size, and induce regeneration of viable myocardium [153].

Other Cardiac Stem Cells

Sca-1$^+$ CSCs are an alternative adult CSC. Evaluation of the corresponding human cell is limited by the absence of the Sca-1 antigen in humans [154]. Lineage fate-mapping studies in mice indicate that Sca-1$^+$ CSCs likely represent a subpopulation of pdgfrα^+ adult epicardial progenitor cells that, similar to c-Kit$^+$ CSCs, minimally contribute to endogenous cardiomyocyte regeneration in response to myocardial damage [60,69,70]. The Isl-1$^+$ progenitors

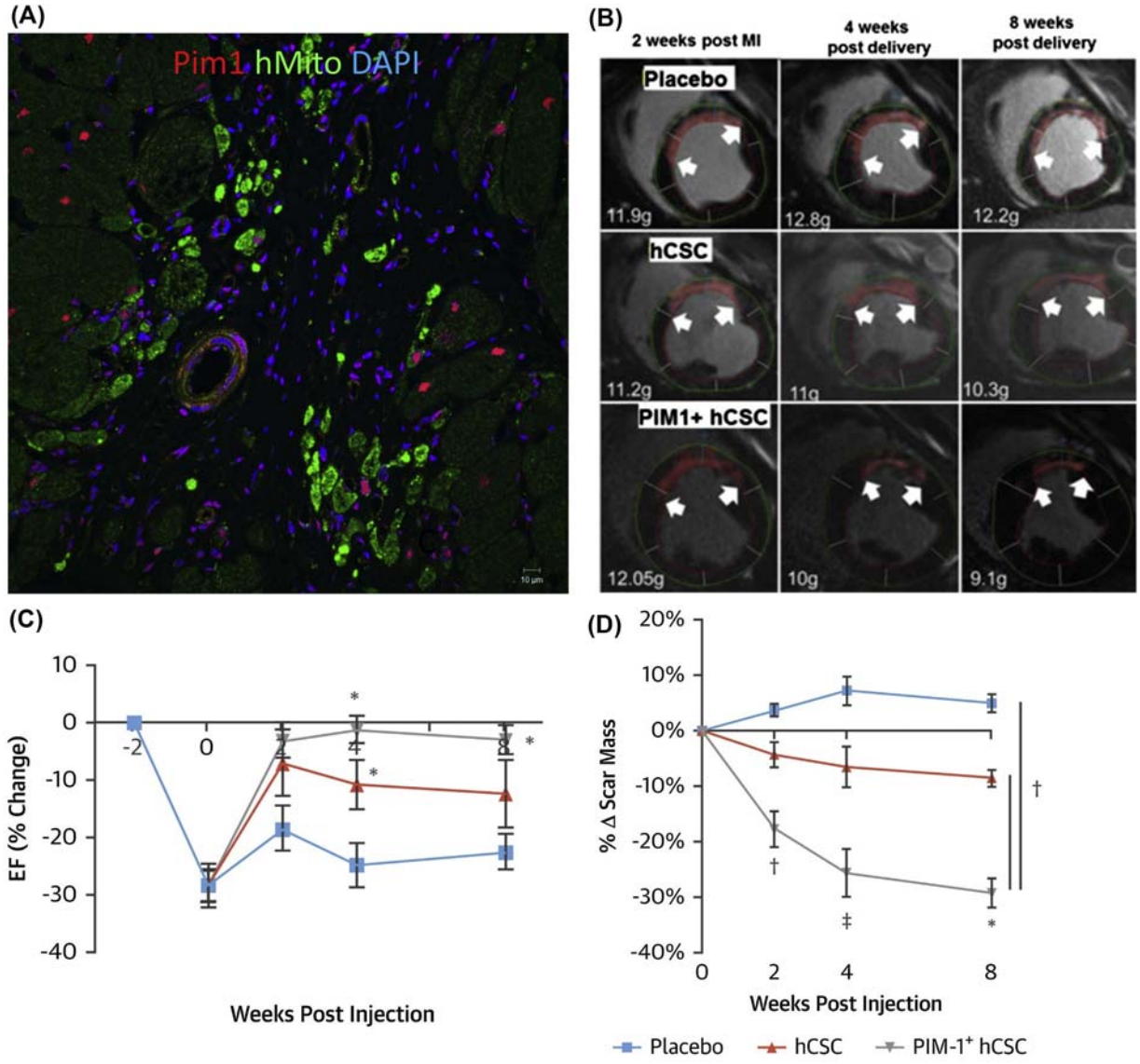


FIGURE 17.11 Genetic engineering of adult cardiac stem cells (CSCs) to enhance their therapeutic potential in response to myocardial damage. (A) Representative confocal microscopy image illustrating engraftment of human CSCs (green fluorescence), genetically engineered to overexpress the proto-oncogene protein kinase Pim1 (red fluorescence), in a porcine heart with experimental myocardial infarction. (B) Representative examples of delayed enhancement of the myocardium using gadolinium cardiac magnetic resonance images from placebo, CSC, or Pim1-overexpressing human CSC (hCSC)-treated animals depict the progress of the delayed enhancement at the same location throughout different points during the study. (C, D) At 4 and 8 weeks postinjection, both cell-treated groups exhibited improved ejection fraction and decreased scar formation compared with placebo. However, the PIM1-engineered CSCs exhibited three times greater antifibrotic effects compared with conventional CSCs. *MI*, myocardial infarction. *Adapted from Kulandavelu S, Karantalis, V, Fritsch, J, Hatzistergos, KE, Loescher, VY, McCall, F, Wang, B, Bagno, L, Golpanian, S, Wolf, A, et al. Pim1 kinase overexpression enhances c-kit+ cardiac stem cell cardiac repair following myocardial infarction in Swine. J Am Coll Cardiol 2016;68:2454–64. https://doi.org/10.1016/j.jacc.2016.09.925.*

have been isolated only from embryonic and very young murine cardiac tissues that do not exceed age 8 days [4]. Early studies suggested that they possibly represent cell remnants from the secondary heart field during embryonic development [26,53]. However, later studies indicated that a neural crest origin is also possible [56]. Isl-1^{+} cardioblasts have not yet been isolated from human heart tissue, but they have been derived in the laboratory from human pluripotent stem cells [53]. Genetic lineage-tracing experiments in mice have failed to document the contribution of postnatal Isl-1^{+} cardioblasts in endogenous cardiomyocyte regeneration in response to damage [59]. Abcg2^{+} SP cells are a cardiac precursor cell population found in rodent hearts [148]. However, although immunohistological studies have documented the existence of MDR-1^{+} and Abcg2^{+} cells in the postnatal human heart [41], isolation and expansion of these cells into therapeutic quantities have yet to be reported.

METHODS FOR EXPANSION OF ADULT CARDIAC STEM CELLS

Two general mechanisms have been employed to isolate and expand CSCs. These are antigen-panning techniques to identify cells such as c-Kit, sca-1, or abcg-2 or direct cell amplification. In the latter case, cells can be readily amplified from cardiac explants. These have been termed cardiospheres (CSs).

Cardiosphere Forming Cells

There have been several attempts to culture cells from the adult heart. Messina and colleagues reported on "cardiospheres" (CSs) structures akin to "neurospheres: [149]. CSs are self-aggregating structures arising from cultured cardiac cells and represent a heterogeneous population possessing cardiopoietic properties in vitro and in vivo [63]. CSs can be derived from human biopsies and are reported to contain $CD105^{+}$, c-Kit+, Sca-1+, and Flk1+ cells. When cocultured with neonatal rat cardiomyocytes, they transdifferentiate into cardiomyocytes, demonstrating calcium transients synchronous among the myocytes as well as spontaneous action potentials. When injected into infarcted rat hearts, ventricular function improved. In a study by Johnston et al., CSCs were injected into a porcine model of MI and were shown to abbreviate but not reverse progressive cardiac remodeling [155].

Cardiospheres represent a potential therapeutic opportunity because of their ability to expand potential cardiac stromal cells from smaller amounts of myocardial tissue, such as a cardiac biopsy. Cardiospheres are incompletely characterized, and whether they offer regenerative capacities superior to BM-derived or other stem cells will require formal testing. CS-derived cells (CDCs) have entered clinical trials [156]. The Intracoronary Cardiosphere-Derived Autologous Stem Cells to Reverse Ventricular Dysfunction trial was a phase I randomized, dose-escalation study of the safety and efficacy of intracoronary delivery of cardiosphere-derived stem cells in patients with ischemic LV dysfunction and a recent MI [156]. Patients who received infusion of CDCs exhibited an increase in viable heart mass, regional contractility, regional systolic wall thickening, and a reduction in scar mass. However, compared with c-Kit^{+} CSCs [153], CDC infusion did not improve LV end-systolic volume, end-diastolic volume, or EF [156]. Although early reports suggested that infused CDCs improve heart function via trilineage differentiation into cardiovascular derivatives [63], more recent studies indicate that the beneficial effects of CDC therapy are likely through paracrine signaling, such as secretion of exosomes [93].

COMBINED STEM CELL THERAPEUTICS

An intriguing effect of MSC therapy in response to ischemic heart damage is their ability to stimulate endogenous CSCs [10,90,92,97,129]. This observation has led to the hypothesis that mixing the two stem cell classes together may yield additive effects compared with each cell type alone. This hypothesis was explored in two proof-of-concept preclinical studies in a porcine model of experimental MI [80,113]. In the first study, intramyocardial injection of 200 million human MSCs combined with one million human CSCs in immunosuppressed pigs 2 weeks after MI resulted in twofold greater reductions in scar size and an increase in cell engraftment by sevenfold compared with treatment with each cell type alone. Moreover, these effects were accompanied by significant improvements in diastolic and systolic function (Fig. 17.12) [80]. The second study tested the combination of autologous MSCs and CSCs in a porcine model of chronic MI, to model its clinical application for HF more accurately. Again, combined transplantation of MSCs and CSCs resulted in significant reductions in scar size and increased viable tissue and cardiac function, which were accompanied by significant increases in endogenous cardiomyocyte mitosis [113]. These promising preclinical results are being tested in the Combination of Mesenchymal and C-kit+ Cardiac Stem Cells as Regenerative Therapy for Heart Failure trial, a multicenter phase II, randomized, placebo-controlled clinical trial designed to assess the feasibility, safety, and effect of autologous MSCs and CSCs both alone and combined compared with placebo as well as each other, administered by transendocardial injection in subjects with ischemic cardiomyopathy (NCT02501811). This ongoing trial, which is conducted by the National Heart, Lung, and Blood Institute's Cardiovascular Cell Therapy Research Network, will examine 144 patients. The results of this trial are highly anticipated and should provide future direction to the field.

Another promising approach of combined cell therapeutics is the mixture of adult BM MSCs with M2 macrophages [157]. Although the exact mechanism(s) of action remain unknown, it is hypothesized that this strategy combines the salutary effects of MSCs with the antiinflammatory actions of M2 macrophages to stimulate heart

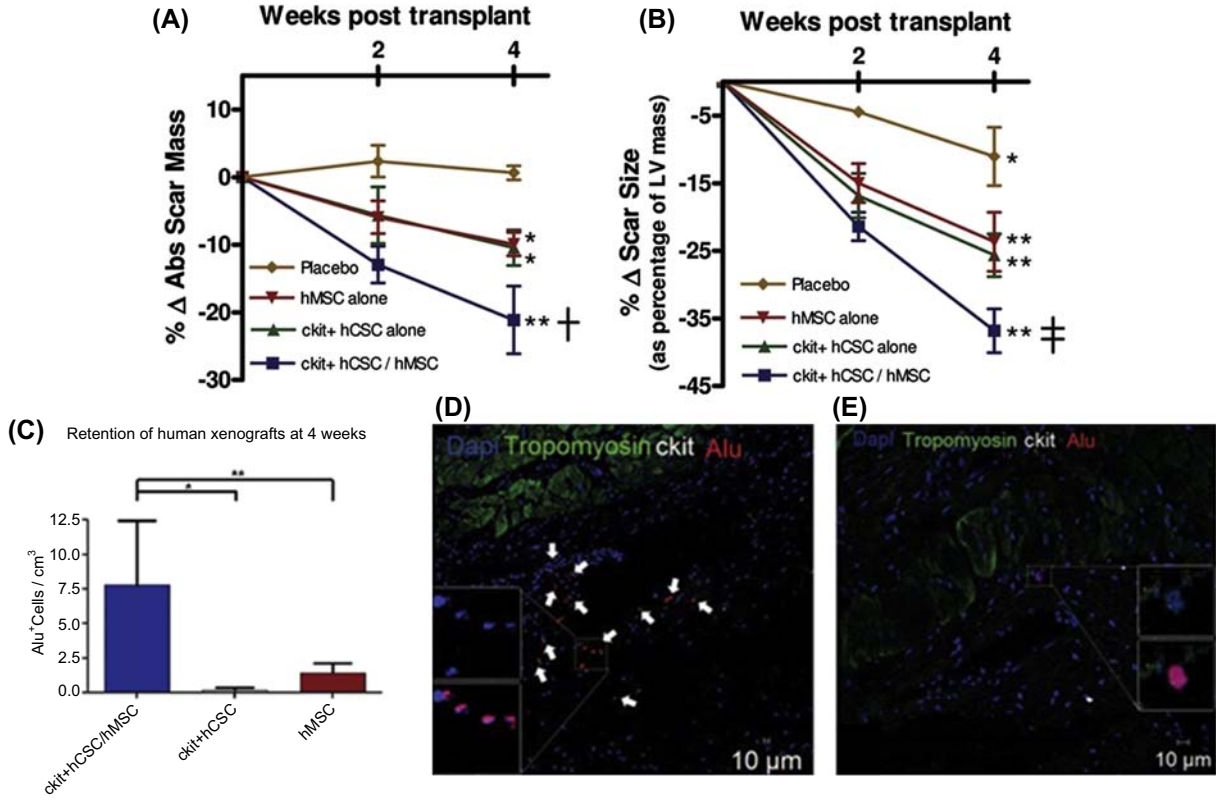


FIGURE 17.12 Combination cell therapy with mesenchymal stem cells (MSCs) and cardiac stem cell (CSCs). (A, B) Infarct size reduction after human CSC (hCSC) and bone marrow human MSC (hMSC) therapy in a xenogenic porcine model of myocardial infarction. Reduction in (A) absolute (Abs) infarct size and (B) infarct size as a percentage of left ventricular mass shows that all stem cell–treated pigs had reduced infarct size compared with placebo and combination therapy had substantially greater scar size reduction compared with either hMSC alone or hCSC alone. (C) Retention of Alu^+ human stem cells was sevenfold higher when hCSCs and hMSCs were injected together compared with either cell type administered alone. Immunohistochemical-stained images showing clusters of Alu-positive human stem cells (*white arrows*) engrafted in the (H) infarct territory and (I) vasculature at 4 weeks posttransplantation. *Adapted from Williams AR, Hatzistergos KE, Addicott B, McCall F, Carvalho D, Suncion V, Morales AR, Da Silva J, Sussman MA, Heldman AW. Enhanced effect of combining human cardiac stem cells and bone marrow mesenchymal stem cells to reduce infarct size and to restore cardiac function after myocardial Infarction Clinical perspective. Circulation 2013;127:213–23.*

repair. This hypothesis was initially tested in two open-label phase 2A trials, the Use of Ixmyelocel-T (Formerly Catheter-based Cardiac Repair Cell [CRC]) Treatment in Patients With Heart Failure Due to Dilated Cardiomyopathy [158]. In these trials, a total of 61 patients with congestive HF caused by ischemic or nonischemic cardiomyopathy were randomized to be treated with standard of care or transendocardial injections of ixmyelocel-T, an autologous BM cell product that underwent a 200-fold enrichment in $CD45^+/CD14^+$ M2 macrophages and a 50-fold enrichment in $CD90^+$ MSCs before transplantation. The results showed that the combination cell therapy significantly reduced major adverse events and improved symptoms in patients with ischemic heart disease but had no benefit in patients with nonischemic heart disease. These findings prompted the Ixmyelocel-T Administered Via Transendocardial Catheter-Based Injections to Subjects With Heart Failure Due to Ischemic Dilated Cardiomyopathy ix study, a randomized, double-blind, placebo-controlled phase 2B trial in which 126 patients with HF resulting from ischemic dilated cardiomyopathy were randomized to ixmyelocel-T ($n = 66$) or placebo ($n = 60$) [157]. The study is considered successful because it met its prespecified primary efficacy end point: a composite measure of the total number of clinical cardiac events as defined by all-cause deaths, cardiovascular admissions to hospital, or unplanned outpatient and emergency departments visits to treat acutely decompensated HF during the 12 months after administration of study treatment. However, the improved clinical outcome was not accompanied by significant improvements in LV function. Furthermore, only 109 of the 126 patients who were enrolled were finally included in the per-protocol primary efficacy analysis (58 in the ixmyelocel-T group and 51 in the placebo group), whereas the remainder of the patients were excluded for a variety of reasons including protocol violation, inadequate cell product, or catheterization/injection procedure-related adverse events that occurred within 7 days of treatment.

CONCLUSIONS

We have witnessed the rapid development of mechanistic and clinical trial support for the notion of a new paradigm in treating heart disease based on cellular therapeutics. Several important insights have emerged supporting this paradigm: (1) the discovery that the heart has capacity for self-renewal and harbors reservoirs of precursor cells that can be tapped or manipulated for therapeutic benefit; (2) remote cell sources, notably BM, also contain cellular constituents with profound therapeutic potential; and (3) cell-based therapies have a remarkable safety profile and can be delivered by diverse methodologies that range from intravenous administration in AMI to directed catheter-based injection systems in chronic HF. An emerging database of clinical trials and fundamental scientific enquiry provides a foundation for this strategy and holds promise for a treatment strategy aimed at a key pathophysiologic target in heart disease, that of ventricular remodeling.

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CHAPTER

18

Skeletal Muscle Stem Cells

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INTRODUCTION

Skeletal muscle is essential for all voluntary contractile functions, including those of the limb, extraocular muscles, tongue, intercostal muscles, diaphragm, and sphincter. As a tissue, skeletal muscle comprises a major part of the human body, accounting for 28%–54% of total body mass depending on gender, age, and physical fitness [1]. It is composed of bundles of muscle fibers, or myofibers, that contract to generate force. Myofibers themselves are composed of contractile myofibrils, which are made up of hundreds to thousands of fused, postmitotic, terminally differentiated muscle cells. Each myofiber is sheathed by a cell membrane called the sarcolemma. Postnatal skeletal muscle growth, maintenance, and regeneration depend on a population of tissue-specific muscle stem cells (MuSCs) present in skeletal muscle, also known as satellite cells. Satellite cells are dedicated MuSCs that reside atop the myofiber between the sarcolemma and the basal lamina of the muscle myofiber [2]. Extensively characterized both functionally and molecularly as bona fide MuSCs, satellite cells have been shown to have a role in maintaining muscle mass in homeostasis and to be crucial for regenerating skeletal muscle in response to acute or chronic injury [3–8]. Other cell sources that contribute to muscle regeneration include muscle interstitial cells, fibrogenic/adipogenic progenitors (FAPs), blood vessel–associated mesoangioblasts, pericytes, and mesenchymal stem cells.

At homeostasis, normal adult skeletal muscle tissue undergoes relatively little turnover compared with other regenerative tissues such as the blood and skin, because only about 1%–2% of myonuclei are replaced weekly, [9]. Evidence has shown that the rate of myofiber remodeling may be higher, with 10% of myofibers showing contribution from a satellite cell per week [10,11]. In response to injury, healthy adult skeletal muscle has a remarkable ability to regenerate [12]. During regeneration, injury to myofibers first leads to a rapid immune response and an infiltration of inflammatory cells [13]. MuSCs are rapidly activated and proliferate as transient amplifying progenitors called myoblasts. A small proportion of activated MuSCs self-renew to replenish the stem cell pool. As regeneration proceeds, proliferating myogenic progenitor cells expand, differentiate, and subsequently fuse with existing myofibers or each other to form de novo myofibers. The newly formed multinucleated myofibers fuse into preexisting myofibers to replenish skeletal muscle mass and contractile function, thus repairing the injured tissue.

Inherited muscular dystrophies and noninherited muscle wasting diseases are characterized by extensive muscle degeneration, and thus are candidates for cell-based regenerative therapies. Heritable muscular dystrophies lead to progressive and often fatal muscle wasting. The most common form of muscular dystrophy is Duchenne muscular dystrophy (DMD), a severe X-linked recessive disorder that affects 1 in 3500 males [14]. DMD is caused by mutations in the dystrophin gene. The dystrophin gene is greater than 2.22 megabases and is one of the largest known genes in the human genome. The large dystrophin protein, which is 427 kDa, links the actin cytoskeleton network within the muscle myofiber to extracellular matrix (ECM) proteins of the basal lamina via the membrane-spanning dystroglycan complex [15,16]. Mutations in dystrophin result in a weakened sarcolemma that leads to loss of myofiber function and widespread muscle degeneration and necrosis. Patients with DMD progressively lose skeletal muscle mass, function, and regenerative potential, in part owing to the reduced stem cell proliferative capacity of MuSCs caused by the excessive demands of constant injury and regeneration [17–20]. As the disease progresses through early adulthood, patients with DMD develop severe muscle deterioration with the exception of facial muscles, increased

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connective and fibrotic tissue deposition, loss of mobility, and kyphosis; the disease culminates in an untimely death from cardiorespiratory failure.

Treatments for muscular dystrophies and muscle wasting conditions such as aging-associated sarcopenia and pathology-associated cachexia have the challenge of rescuing defective postmitotic myofibers [21,22]. Although many pharmacologic and genetic therapies have been developed to minimize inflammation and support muscle regeneration for these diseases, they have met with little long-term clinical success, especially for the most devastating degenerative conditions [23,24]. Therefore, increased focus has been placed on developing regenerative strategies that target MuSCs to provide more effective therapies. There has been substantial progress in elucidating the molecular signals and myogenic gene regulatory mechanisms that underlie the regenerative capacity of MuSCs [25,26]. Thus, here we highlight the discovery of satellite cells as an endogenous source of MuSCs that contribute to adult muscle regeneration. Advances in regulating MuSC activation and self-renewal by biochemical and biophysical components of the MuSC niche microenvironment have been substantial. We evaluate the potential of satellite cells and other cell sources with myogenic regenerative properties for cell-based therapy for human muscle diseases. We also describe how technological advances offer promise for advancements in targeting endogenous stem cells in muscle wasting disorders or for cell therapy of heritable muscular dystrophies.

SATELLITE CELLS ARE MUSCLE STEM CELLS

To be considered a tissue-specific stem cell, cells must be able to (1) proliferate in a self-renewing manner that yields at least one daughter cell with a stem cell profile and (2) generate progeny capable of differentiating into all of the specialized cells of that particular tissue. For some tissues, stem cells must develop into a set of multipotent transient progenitors that can produce the complete diversity of terminally differentiated cells that make up the tissue. For example, a single hematopoietic stem cell (HSC) is capable of generating lymphoid, myeloid, and erythroid progenitors, which then generate all of the cells in the blood compartment. In contrast, MuSCs must give rise to only one single terminally differentiated cell type, the multinucleated myofiber. Consequently, a bona fide MuSC must be a self-renewing unipotent cell that is also capable of generating progeny that fuse with existing myofibers or generate new myofibers. The requirement of MuSCs to self-renew implies that MuSC division results in specialized progenitors in a manner that does not deplete the stem cell pool. This property distinguishes MuSCs from their myoblast progeny, which are able to undergo only a limited number of divisions before they undergo myogenic differentiation, thus causing their depletion. The long-term self-renewal characteristics of MuSCs confer the ability to contribute to myogenesis throughout the life span of an organism. Moreover, the stem cell capacity of MuSCs fulfills the requirements of an ongoing, continuous therapeutic cell source for long-term skeletal muscle regenerative medicine applications. In this section, we describe the molecular and functional characteristics that define MuSCs and how these can be combined to track prospectively isolated, transplanted cells and monitor their regenerative capacity.

THE MOLECULAR CHARACTERISTICS OF MUSCLE STEM CELLS DURING MYOGENESIS IN REGENERATION

Since their first identification by electron microscopy based on their anatomical position [2], our understanding of the molecular characteristics of satellite cells has greatly increased. Quiescent satellite cells, which have been shown to be MuSCs, are defined by the expression of the essential paired-box transcription factor *Pax7* [27,28]. Mice that lack PAX7 protein develop normally in utero, but only about half of expected homozygotes live to birth. Newborns that escape this early postnatal lethality typically survive only until about age 3 weeks and show approximately 50% reduction in muscle mass with vastly decreased numbers of satellite cells [28,29]. Conditional ablation of $PAX7^{+}$ cells in the adult mouse leads to acutely impaired muscle regeneration, including decreased myofiber area and increased fibrosis. Knockout of *Pax7* in isolated MuSCs in vitro results in cell-cycle arrest and premature differentiation [27]. In the diaphragm muscle, the paired box transcription factor PAX3 is constitutively expressed in satellite cells; however, this factor is transiently expressed only in hind-limb muscles, and its role as an essential factor in satellite cell maintenance is unclear [30–33]. Functionally, skeletal muscle satellite cells possess an intrinsic stem cell capacity; that is, they are able to develop into muscle and repopulate the stem cell niche independently.

After injury, PAX7^{+} MuSCs become activated and expand. As part of their stem cell identity, MuSCs undergo asymmetric cell divisions to generate both transiently amplifying progenitors that are committed to differentiation and new MuSCs to replenish the stem cell pool [34,35]. The discovery of the basic helix-loop-helix (bHLH) family began with MYOD1 and resulted from a clever strategy derived from the observation that treatment of fibroblasts with 5-aza-C yielded myogenic and adipogenic cells [36]. Upon activation, myogenic progenitors express a series of myogenic regulatory factors (MRFs), bHLH transcription factors that upregulate genes required for differentiation into muscle myofibers (Fig. 18.1E). The dynamics of these transiently amplifying progenitors have been characterized for the first time in vivo via multidimensional single-cell mass cytometry (CyTOF) [37].

In development, *Myf5* is the first bHLH factor to be expressed, and this transcription factor activates downstream myogenic genes in MuSCs and acts as a chromatin modifier of myogenic genes [38]. Lineage tracing experiments show that about 90% of all PAX7^{+} MuSCs express or have expressed the *Myf5* gene at some point, which indicates that it can lead to both stem cell self-renewal or differentiation [34]. Studies have shown that *Myf5* messenger RNA

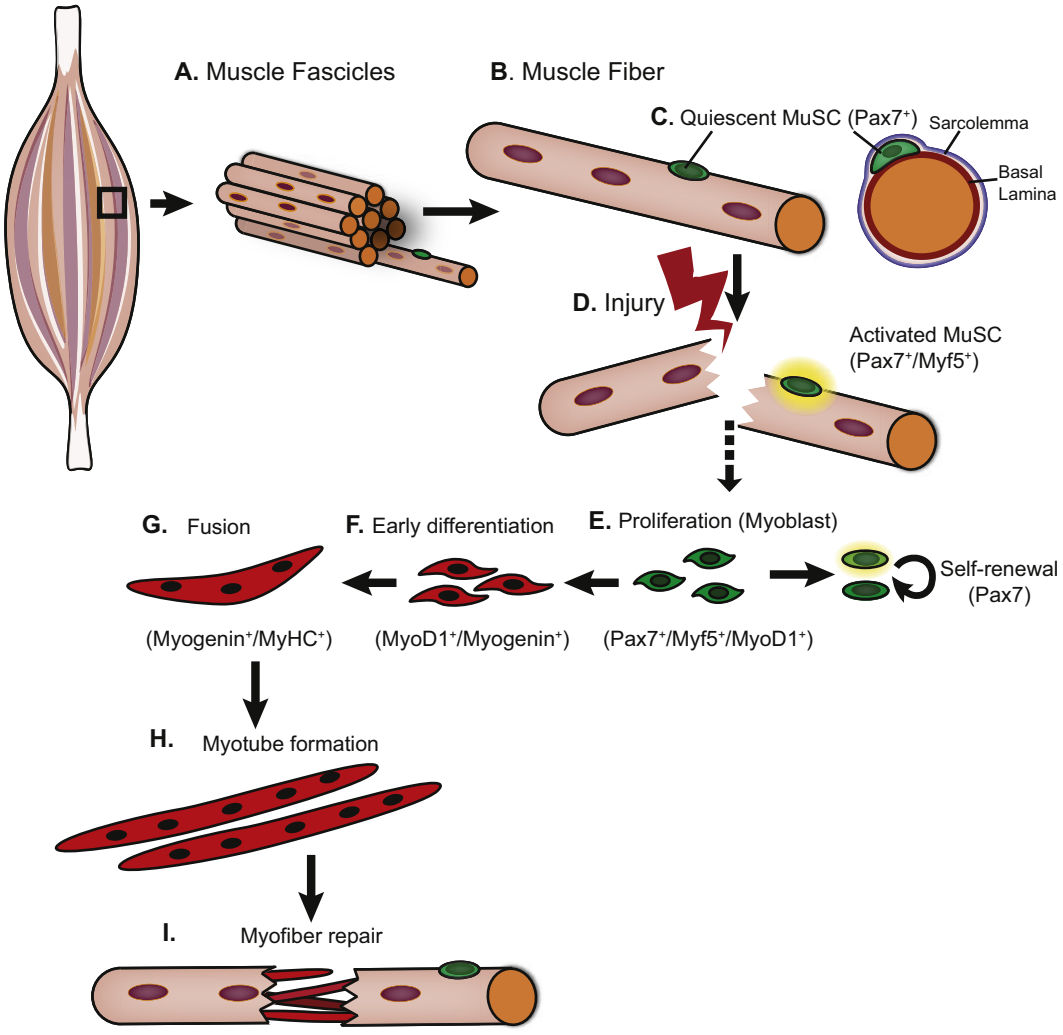

FIGURE 18.1 **Myogenesis during muscle regeneration.** Muscle is a primarily postmitotic tissue that consists of fused muscle cells. Muscle consists of bundles of (A) fascicles, which are in themselves composed of bundles of (B) muscle fibers. Muscle fibers are contractile, multinucleated nucleated cells formed from the fusion of myoblasts. They are sheathed by a cell membrane called the sarcolemma. (C) Muscle stem cells (MuSCs), which are not fused, reside atop the fiber between the sarcolemma and the basal lamina of the muscle fiber. They are maintained in their quiescent state by the expression of transcription factor Pax7. (D) Upon injury, MuSCS become activated and express Myf5. (E) During repair, activated MuSCs expand in the form of transiently proliferating progenitors known as myoblasts. Myoblasts downregulate Pax7 and express MyoD1. A fraction of activated MuSCs self-renew to repopulate the stem cell. After proliferation, myoblasts exit the cell cycle and (F) begin early differentiation, which is marked by expression of the transcription factor myogenin. (G) These cells eventually fuse together and form a multinucleated cell. Myosin heavy chains (MyHC) are expressed at this time. Multinucleated cells fuse to themselves to form (H) mature myotubes that eventually form the (I) new contractile myofibers at the end of regeneration.

(mRNA) is sequestered in quiescent satellite cells in messenger ribonucleoprotein (mRNP) granules, which suppress translation [39]. This sequestration of *Myf5* mRNA allows MuSCs to be poised for activation and entrance into the myogenic program while maintaining quiescence. After activation, mRNP granules become dissociated, allowing *Myf5* mRNA to be translated immediately and MYF5 protein to accumulate rapidly. Later during development, MYF5 function has redundancy with bHLH regulatory factor MRF4 [40], which promotes early muscle formation. In adult regeneration, MRF4 inhibits muscle growth and hypertrophy [41].

MuSC activation is later marked by the bHLH factor, MYOD1. MYOD1 is a potent transcriptional activator of myogenesis [42]. MYOD1 shares many similar genomic targets with MYF5 and can rescue myogenesis in the absence of MYF5 [43]. Because of their largely redundant function, knockout of either MYOD1 or MYF5 is not associated with skeletal muscle defects [44], but MYOD/MYF5 double knockouts lack expression of myogenic genes and have no discernible formation of muscle myofibers [43]. In the presence of high levels of mitogens, MYF5 and MYOD1 are highly expressed and define commitment to a myogenic cell fate but they do not activate the expression of differentiation genes. In particular, MYOD1 is known to associate with histone deacetylase 1 during proliferation to repress myogenic differentiation targets [45]. Removal of serum in vitro induces cell cycle exit, which is driven in part by MYOD1 upregulation of p21, a cyclin-dependent kinase inhibitor [46]. After cell cycle exit, MYOD1 associates with histone acetyl-transferases to induce acetylation and expression of myogenic differentiation genes [47,48]. The final stage of myogenesis is driven by expression of the transcription factor Myogenin (MYOG), the canonical marker of terminal differentiation (Fig. 18.1F) [49].

Myosin heavy chain isotypes distinguish fast and slow myofibers that differ in their stem cell and regenerative properties. They are expressed during late differentiation and upon fusion of myoblasts [50] (Fig. 18.1G). These proteins are highly conserved, actin-based motor proteins that use energy from adenosine triphosphate (ATP) to generate contractile force in mature myofibers [51]. Myosins are upregulated as fusion proceeds, forming multinucleated myotubes (Fig. 18.1H) that then fuse to each other to form myofibers and repair the injured tissue at the end of regeneration. A number of different myosin heavy chain isoforms define distinct fiber types in the adult muscle: I, IIA, IIB, and IIX [52]. Type I fibers are slow-twitch and mitochondria-dense and derive ATP primarily from oxidative phosphorylation of fat. These fibers are also known to have the highest density of $PAX7^+$ stem cells per fiber [53]. Rich in myoglobin, type I fibers can maintain contractile activity and are resistant to fatigue. Type II fibers rely on glycolysis to generate ATP rapidly. Type IIA fibers use oxidative glycolysis and have more mitochondria than type IIB fibers, which primarily use anaerobic glycolysis. Type IIB fibers are the fastest but generate force for the shortest time and are the first to be affected in DMD [54]. Type IIX fibers are mixed between IIA and IIB [52].

Expression of myosin isoforms is highly dynamic during development and regeneration [55] as well as in vitro differentiation [50]. Embryonic myosin isoforms, which typically are expressed during development, are reexpressed during early differentiation or regeneration [56,57]. Neonatal fast and slow myosins precede adult myosin isoform and can be distinguished from the adult myosin isoforms at the end of regeneration by monoclonal antibodies [58,59]. The functional basis for the observed developmental progression of myosin isoforms remains unknown. Reestablishment of fiber types during late regeneration and postnatal development is independent of cell lineage [60,61] and primarily caused by innervation [55,62,63].

The search for mediators of myogenic fusion, a step essential to muscle development and regeneration, has been under way for decades. A number of integral components implicated in myofusion have been implicated using a β-galactosidase (β-gal) complementation method [64], including neural cell adhesion molecule (NCAM) [65] and N-cadherin [66]. Additional studies pointed to α4-integrin [67], M-cadherin [68], vascular cell adhesion molecule (VCAM) [69], β1-integrin [70], MOR23 [71], and the metalloproteinase/disintegrin meltrin-α [72] as mediators of fusion. However, the proteins that are essential to muscle fusion been definitively identified only recently. The muscle-specific gene Myomaker (TMEM8c) [73] is absolutely required for fusion in vitro or during development. However, Myomaker alone does not suffice for fusion to occur, because overexpression of Myomaker causes fusion of fibroblasts with myoblasts but not fusion between myoblasts [73]. Myomaker's partner has been identified as the transmembrane protein Myomixer [74]. Although the molecular components required for muscle fusion continue to be discovered, the mechanism of fusion is still under investigation.

FUNCTIONAL CHARACTERISTICS OF MUSCLE STEM CELLS

One of the first indications that $PAX7^+$ satellite cells are MuSCs was established by transplantation studies. In these studies, satellite cells juxtaposed to single fibers were genetically labeled by Myf5-β-gal, and single fibers, which were confirmed to contain resident satellite cells, were transplanted into damaged recipient wild-type

(WT) muscles [5]. The donor myofibers resulted in new myofibers, and associated satellite cells were able to self-renew and develop into new satellite cells in the niche position in the recipient animals [5]. However, the use of entire myofibers for transplantation did not demonstrate definitively that $PAX7^+$ satellite cells could become the entire myogenic lineage on their own. The intrinsic stem cell capacity of satellite cells alone was demonstrated through later single-cell isolation strategies. Satellite cells were genetically labeled to express reporter genes such as green fluorescent protein (GFP) or β-gal and isolated from single-cell muscle digests or mechanical trituration of isolated myofibers. These cells were transplanted by intramuscular injection into recipient muscle of regeneration-deficient mice [30,75,76].

In transplantation assays to evaluate MuSC properties, recipient mouse hind-limb muscles such as the tibialis anterior are often irradiated pretransplantation to limit the contribution of endogenous stem cell populations, as in HSC transplantation scenarios. Sometimes mice are also injured acutely before and/or after transplantation, either with a chemical toxin such as notexin or by direct tissue injury (e.g., freeze-probe application), to assess the stem cell and proliferative capacity of donor cells. Alternatively, satellite cells are transplanted into recipients with a muscle regenerative defect, such as the *mdx* mouse model of DMD, to assess the ability of the stem cells to meet a chronic regenerative demand. Through these approaches, multiple investigators have demonstrated that $PAX7^+$ satellite cells are able to contribute to the regeneration of recipient myofibers, repopulate the myofiber membrane-defined satellite cell niche, and expand and self-renew upon activation by injury [5,30,34,75–77]. Using such regimens, isolated labeled satellite cells were shown to contribute directly to myofibers as well as the resident stem cell population.

ISOLATION OF MUSCLE STEM CELLS

A number of strategies have been developed to isolate MuSCs prospectively. Fluorescence-activated cell sorting is used to identify MuSCs based on their unique profile of surface markers. Prospective isolation strategies routinely employed a combination of markers, often including the absence of lineage markers such as CD31, CD11b, CD45, and Sca1 to obtain a highly enriched cell population. For mouse satellite cells, α7-integrin (ITGA) [77], which is also present on myoblasts [78], is the most commonly used marker for isolation in conjunction with β1-integrin [34,75], CD34 [76], CXCR4 [75], VCAM1 [79], syndecan (SDC)-3/4 [6], ABCG2 [6], and c-met [80]. Additional sorting strategies have used lineage depletion combined with the antigen for the SM/C-2.6 monoclonal antibody [81], and the calcitonin receptor CALCR [82]. Other mouse MuSC prospective isolation strategies use transgenic fluorescent protein reporters under the control of promoter elements of satellite cell-associated transcription factors such as *Pax7-eGFP* [83], *Pax7-ZsGreen* [84], and *Myf5-Cre/ROSA26-YFP* [34]. These genetically labeled models have been used to distinguish subpopulations further. Two cell surface markers, CD9 and CD104, identified by single-CyTOF profiling of injured muscles, enabled in vivo identification and prospective isolation of stem and progenitor cells [37]. It is likely that none of these markers encompasses all MuSCs, because there is known to be heterogeneity within MuSC populations.

Studies have uncovered cell surface markers that enrich for human satellite cells. Human satellite cell markers have long been sought, because human markers do not completely correspond to those on well-defined mouse satellite cells. For example, unlike mouse satellite cells, human satellite cells are not $CD34^+$ [85,86]. In addition, PAX7 expression does not appear to be necessarily restricted to satellite cells in human muscle but is also expressed by progenitors [87]. Human myogenic cells were first prospectively isolated using a monoclonal antibody to an antigen identified as NCAM [88]. $CD133^+$ and culture-expanded human muscle–derived myoblasts have also been shown to have engraftment potential and generate satellite cells [89,90]. However, the xenotransplantation efficiency of these cells is low. Studies have found cell surface markers of fetal [91] and adult [92], $PAX7^+$ muscle satellite cells with MuSC function. Fetal satellite cells were identified as negative for blood and fibroblast markers CD45/CD11b/GlyA/CD31/CD34, and intermediate for NCAM (CD56) and high ITGA expression. These $CD56^{Int}ITGA7^{hi}$ show bipotent myogenic and osteogenic activity with no adipogenic capacity in vitro and engraft when transplanted into mouse muscles. However, the same markers do not mark adult satellite cells with engraftment capacity. Adult MuSCs can be isolated by $CD31^-/CD45^-/$β1-integrin $(CD29)^+/NCAM(CD56)^+$ expression [92] or $CD31^-/CD45^-/$β1-integrin and epidermal growth factor receptor [93]. In addition, CD82 and CD318 have been identified as marking myogenic cells with high engraftment potential [94,95]. These cells express PAX7 and can both contribute to myofibers and repopulate the satellite cell niche when transplanted into mouse muscle. Similar to mouse MuSCs, these transplanted human adult MuSCs expand upon reinjury, an indicator of true stem cell capacity.

TRACKING MUSCLE STEM CELL BEHAVIOR THROUGH LIVE IMAGING (BIOLUMINESCENCE IMAGING AND INTRAVITAL IMAGING)

The development of noninvasive imaging techniques has greatly advanced our understanding of MuSCs. Bioluminescence imaging (BLI) enabled the first demonstration that a single satellite cell fulfills the stringent criteria of a MuSC [76]. BLI has been highly useful in studies to establish the regenerative potential of other tissue-specific stem cell types. These studies showed that only 4% of 144 mice injected with single MuSCs exhibited a positive BLI signal, a result that was difficult to ascertain by classical histological methods. Similarly, the ability to track the behavior of small numbers of transplanted cells in vivo (<10) revealed the intrinsic deficit of aged MuSCs to engraft, a finding not previously apparent through transplantation of larger cells numbers (>100) [26].

BLI uses luciferase-expressing satellite cells that are often colabeled with GFP, which allows for dynamic monitoring of the cells as they contribute to tissue regeneration, as well as end-point immunohistological analysis (Fig. 18.2). Temporal assessment of stem cell behavior upon stimulation with a drug, hormone, or growth factor is also possible by BLI, because the same mice can be imaged noninvasively repeatedly over time, whereas classical histological analyses require killing the mice [96]. BLI revealed that after reinjury, the progeny of transplanted satellite cells divide exponentially and then cease to divide, reaching a plateau upon achieving homeostasis when no more stem cells are required [76]. By contrast, 90% of myoblasts without self-renewal capacity die upon injection and no increase in numbers is observed over time, which explains in part why clinical trials with these cells have been disappointing [85,97,192]. Furthermore, it is possible to assess whether transplanted stem cells are transformed, because the exponential proliferation of tumorigenic cells does not cease and a plateau is never achieved [98]. This type of exponential increase is not typically observed with transplanted MuSCs and rhabdomyosarcomas

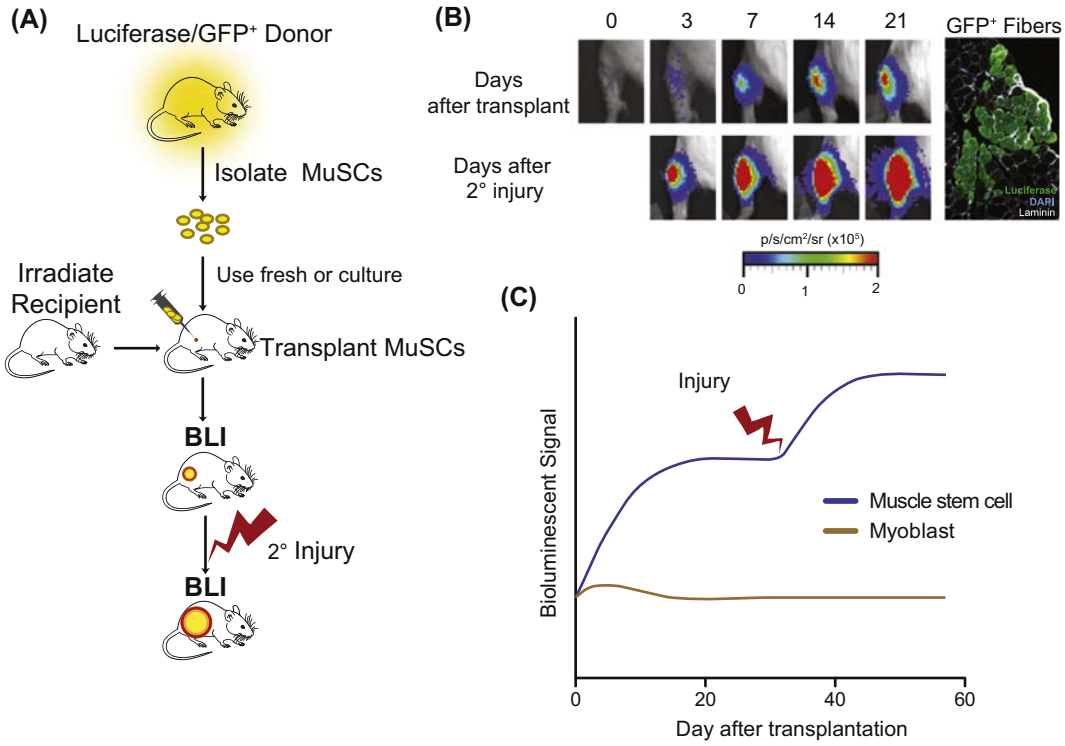

FIGURE 18.2 **Noninvasive bioluminescent imaging (BLI) to visualize the expansion and engraftment of transplanted muscle stem cells (MuSCs) dynamically.** (A) Transplantation scheme for BLI. MuSCs are isolated from a donor animal expressing luciferase and green fluorescent protein (GFP). These cells are then transplanted via intramuscular injection into the hind limb of recipient animals, which are immunocompromised and irradiated to deplete the existing stem cell pool and minimize immune rejection. (B) Example of BLI signal from MuSCs after transplantation and injury. Immunohistological analysis of muscles with transplanted MuSCs show GFP-positive myofibers. (C) Scheme of a theoretical comparison of the engraftment and proliferation of 100 transplanted MuSCs or committed myoblast progenitors. BLI used to track engraftment after injury, as well as expansion after secondary injury. While transplanted MuSCs expand and engraft, myoblasts have poor viability and do not engraft or expand. For MuSC transplantation, the BLI signal reaches a plateau when cells stop proliferating, differentiate, and fuse into mature myofibers. Expansion of transplanted MuSCs that have repopulated the stem-cell niche can be stimulated by injury and tracked by BLI.

are rare in childhood and virtually nonexistent in adulthood. That MuSCs and the muscle tissue microenvironment are nontumorigenic suggests that yet unidentified tumor-suppressive factors are at play.

BLI has been used to track in vivo regeneration and inflammation in skeletal muscle disease states [99,100]. The *Pax7*-driven luciferase reporter mouse (*Pax7Cre^{ER}/LuSEAP*) was created as a "regeneration reporter" line to study the expansion and differentiation of satellite cells during regeneration in vivo [99]. These mice had a low level of luciferase signal at baseline that increased after 3 days of injury by barium chloride injection. At the end of regeneration, once myofibers matured, the luciferase signal decreased. Luciferase-positive myotubes were apparent upon histological analysis, which was consistent with the contribution from luciferase-expressing MuSCs. To analyze the expansion of *Pax7*-expressing MuSCs during chronic muscle regeneration, *Pax7Cre^{ER}/LuSEAP* animals were crossed to dysferlin-deficient *Dysf$^{-/-}$* mice, which are a model of limb girdle muscular dystrophy. *Dysf$^{-/-}$/Pax7Cre^{ER}/LuSEAP* mice showed a persistent increase in luciferase signal over time, indicative of ongoing regenerative activity. Similarly, an inflammation-driven luciferase reporter line (*Cox2$^{Fluc/+}$*) was crossed with the *mdx* mouse model of DMD to analyze muscle inflammation during disease progression in vivo [100]. BLI showed increased inflammation in response to damage by exercise or cardiotoxin injury, and a decrease upon treatment with steroids to reduce inflammation. These studies highlight the promise for future application of BLI for the rigorous quantitative assessment of muscle regeneration in different mouse models of chronic and acute muscle injuries typical of human muscle diseases, as well the efficacy of therapeutic candidates.

Intravital imaging (IVM) with two photon-excitation microscopy has arisen as a technique to study the behavior and function of MuSCs and muscle progenitors during regeneration. This technique provides for high resolution, allows for use of multiple colors, and can be combined with bright-field and secondary harmonic imaging for higher detail of tissue structure in living animals. Two-photon excitation allows for virtual cross-sections of the tissue to be analyzed. A study showed a time course of one million myoblasts transplanted to treat muscle regeneration [101]. Another study used IVM to directly image for the first time MuSCs on uninjured and injured muscle myofibers [102]. Consistent with histological evidence that MuSCs are primarily quiescent, IVM showed neither division nor migration during the imaging period of 3 days. Using the ability of IVM to visualize sarcomere structure through secondary harmonic generation, Webster et al. [102] showed that muscle progenitors divide along "ghost fibers" during regeneration. These ghost fibers are the ECM scaffold surrounding muscle fibers, which remains intact when these myofibers are ablated through injury. Overall, live imaging is a powerful tool for the longitudinal analysis of MuSCs.

REGULATION OF MUSCLE STEM CELLS BY THEIR NICHE

A number of the molecular and biophysical components of the satellite cell niche in mice have been identified that are crucial for directing satellite cell quiescence, activation, migration, and self-renewal. Although the precise roles of these niche factors are still under investigation, current progress is summarized here and detailed in subsequent sections.

Extracellular Matrix Components

The myofiber basal lamina contains a range of ECM components that regulate MuSC fate. These include type VI collagen [103], fibronectin [104,105], tenascin-C (TNC) [106], laminin [107–109], and other proteoglycans and glycoproteins [110]. Collagen VI, which is primarily deposited by muscle-resident fibroblasts, is required for maintaining MuSC self-renewal during homeostasis and injury [103]. Fibronectin is a transient niche component present during regeneration that is secreted by progenitors and promotes self-renewal of MuSCs. It is a preferred ECM substrate for MuSCs, which adhere to fibronectin through integrins and the SDC4–Frizzled-7 (FZD7) complex [105]. Accumulation of fibronectin is thought to be detrimental to differentiation and regeneration. Loss of fibronectin, in particular during aging, leads to a reduction in the stem cell pool after injury [104]. Laminins are expressed on the surface of myotubes in vitro and myofibers in vivo and have been shown to promote migration of myoblasts and preserve differentiation potential [107–109]. A mechanical stress sensor, TNC is expressed in actively remodeling muscle [111]. Knockdown of TNC results in selective atrophy of fast muscle fibers in the adult. In addition, TNC is highly expressed during development and promotes MuSC expansion and regeneration in fetal muscle [106].

Satellite cells express numerous cell-surface molecules that interact with components of the ECM [110,112]. Integrins, which are heterodimers consisting of a α and a β chain, physically tether the satellite cell to the niche and mediate molecular and biophysical signal transduction [113]. MuSCs primarily express the α7/β1 heterodimer.

Evidence has shown that FGF2 signaling is mediated through β1-integrin, which is critical for maintaining MuSC self-renewal and expansion during injury [114]. Another integrin, β3-integrin, has been shown to mediate MuSC differentiation in muscle regeneration [115]. Other important ECM-signaling proteins include heparan sulfate proteoglycans such as SDC3, which promotes self-renewal and inhibits differentiation through Notch activation [116], and SDC4, which mediates asymmetric self-renewal through WNT7A and fibronectin [105].

Molecular Signaling Factors

Growth factors are secreted from systemic and local sources and stimulate satellite cell survival, activation, and proliferation. They can bind to proteoglycans in the myofiber basal lamina or on the satellite cell surface itself to provide a niche repository of signaling molecules. Circulating signaling factors can gain access to satellite cells, because of the close proximity of their niches to both muscle microvasculature [53] and neuromuscular junctions [117], and diffuse through the basal lamina. After injury, inflammatory cells secrete cytokines such as interleukin 1, interleukin 6, and tumor necrosis factor-α that regulate the activation, proliferation, and differentiation of MuSCs [118,119]. The small metabolite prostaglandin E2 has been recently identified as part of the inflammatory wave that is the first response to injury in muscle [120]. The addition of PGE2 transiently leads to a robust increase in muscle stem cell numbers in culture and boosts engraftment of treated cells post-transplantation. Notably, PGE2 delivered directly to injured muscles activates endogenous stem cells within the muscles and markedly accelerates regeneration, suggesting it may have therapeutic value. Other critical circulating factors include fibroblast growth factor (FGF) [114,121–123], hepatocyte growth factor (HGF) [124,125], and insulin-like growth factor-1 (IGF-1) [126,127], among others. FGF stimulates MuSC activation, which is repressed during quiescence by the receptor tyrosine kinase signaling inhibitor Sprouty1 (SPRY1) [128]. HGF, which is also secreted by MuSCs themselves [125], binds to the c-met receptor of MuSCs and promotes activation, proliferation, and migration [124]. IGF-1 is a circulating hormone that stimulates proliferation of MuSCs as well as differentiation. In the transforming growth factor-β (TGF-β) superfamily, myostatin, also known as GDF8, is a secreted factor that has been shown to circulate in serum. Myostatin is a negative regulator of MuSC proliferation as well as muscle hypertrophy, and knockout cattle exhibit profound increases in muscle mass [129–131]. Much attention has been focused on another TGF-β member, GDF11, which is a systemic factor that shares 89% homology with myostatin and signals through many of the same pathways. There have been conflicting reports on the function of GDF11. Some studies have shown that like myostatin, GDF11 inhibits muscle regeneration and decreases MuSC expansion [132], whereas other reports show that it can rejuvenate the regenerative function of aged muscle [133]. The debate remains unsettled, but TGF-β family members remain attractive therapeutic targets.

In addition, MuSCs, progenitors and mature myofibers themselves express factors that influence their own behaviors through juxtacrine signaling. These include ligands for Notch, which maintains the quiescence of adult MuSCs at homeostasis [34,134–136]. Moreover, the Wnt family of proteins is composed of secreted glycoproteins often associated with the ECM. WNT3A acts through canonical Wnt signaling to drive myogenic differentiation [137,138]. WNT7A has been shown to promote symmetric expansion of MuSCs through noncanonical signaling via the receptor FZD7 [21,105,139]. A report showed that the secreted R-spondin protein RPSO1 potentiates these Wnt signaling pathways, promoting WNT3A activation and suppressing WNT7A signaling. Stromal cell-derived factor-1 is secreted by the myofiber and binds the receptor CXCR4 on satellite cells. First identified as a migratory factor during development [140], it regulates the proliferation of progenitor cells and terminally differentiated myoblasts and is necessary for fusion [141,142]. Further elucidation of how satellite cell behaviors are regulated in normal muscle homeostasis and regeneration and are dysregulated in degenerative muscle conditions will be instrumental for the development of methods to maintain and expand MuSCs in culture and therapies directed toward endogenous MuSCs or using transplanted MuSCs.

Biophysical Cues

MuSCs are highly sensitive to changes in the mechanical and topological components of their niche. Cells can respond to extracellular biophysical cues through a variety of mechanotransduction mechanisms [143]. Rigidity of the surrounding matrix, characterized by Young's modulus, is known to direct cultured multipotent mesenchymal stem cells toward myogenic, chondrogenic, adipogenic, or osteogenic cell fates in culture [144]. In muscle, matrix rigidity has been shown to be highly important for maintaining MuSC self-renewal and stem cell functions. In vivo, skeletal muscle stiffness increases during age and disease as a result of increased fibrosis [145–148]. Elevated rigidity of skeletal muscle is apparent in the *mdx* mouse model of DMD, which has an elastic modulus of $E > 18$ kPa

after age 4 months [145,146]. MuSCs plated on hard plastic, which have a Young's modulus of E $\sim 10^6$ kPa, lose stem cell function within 7 days. A hydrogel material with the same rigidity as muscle tissue (E = 12 kPa) promotes cell survival, prevents differentiation, and maintains stem cell function, as measured by transplantation assay [149]. These findings suggest that alterations in the elasticity or rigidity of skeletal muscle tissue may have profound effects on the contributions of satellite cells and myogenic progenitors to muscle regeneration.

Metabolic Cues

Skeletal muscle is a highly metabolic tissue that is crucial for maintaining metabolic homeostasis in the organism and functions as a rheostat for metabolic health. Evidence has shown that metabolic cues are critical regulators of MuSC behavior and function [150]. Quiescent MuSCs have been found to associate closely with capillaries [53]; they have a high expression of oxidative phosphorylation and fatty acid metabolism genes and exhibit evidence of mitochondrial respiration with little anaerobic glycolysis [151]. Upon activation, MuSCs switch to more anaerobic glycolysis, concomitant with downregulation of fatty acid metabolism genes [151] as well as increased autophagy to support bioenergetic demands [152]. During muscle regeneration, the heightened inflammatory state also affects MuSCs outside the direct injury site, inducing cells into an "alert" state poised to enter the cell cycle, characterized by increased metabolic activity and activation of the nutrient-sensing mammalian target of rapamycin complex pathway [153].

The metabolic state of the tissue has important implications for the function of MuSCs. Aged MuSCs, which have reduced function compared with young controls, exhibit mitochondrial dysfunction and an aberrant expression of metabolic genes in mice [154]. Restoration of mitochondrial activity by treatment with nicotinamide riboside, a precursor of nicotinamide adenine dinucleotide (NAD^+), increased MuSC numbers and improved the function of both MuSCs and total muscle in aged mice. NAD^+, a by-product of a number of metabolic processes including the oxidative respiratory chain, has also been shown to be critical for the activation of MuSCs. MuSCs activated by short-term cell culture have decreased NAD^+ levels, concomitant with increased anaerobic glucose metabolism. These metabolic changes lead to increased histone acetylation mediated by decreased activity of the NAD-dependent histone deacetylase SIRT1. Knockout of *Sirt1* in $PAX7^+$ MuSCs results in aberrant activation and increased histone acetylation of quiescent cells, leading to decreased regenerative capacity [151]. Finally, short-term calorie restriction (12 weeks) of young or aged mice has been shown to enhance MuSC numbers and stem cell function, including increased ex vivo myogenic colony-forming capacity and the ability to engraft into recipient (WT) muscle tissue [155]. Caloric restriction also increased mitochondrial capacity and respiration and increased the fraction of MuSCs expressing *Sirt1*. MuSCs isolated from WT tissue and transplanted into calorie-restricted recipients also showed increased engraftment, which demonstrated that the benefits of calorie restriction are extrinsic as well as intrinsic.

In contrast, muscle regeneration is known to be impaired in obesity and type 2 diabetes (T2D) [156,157], conditions that result in muscle-specific metabolic inflexibility and increased mitochondrial stress [158]. Mice given a high-fat diet (HFD) (60% kcal) to induce glucose intolerance have reduced muscle regeneration after either short-term (3–6 weeks) [159] or long-term (8 months) [157] administration of the diet compared with normal diet (10% kcal) controls. These regenerative defects are also cell-intrinsic. MuSCs isolated from mice fed an HFD for 8 weeks have reduced proliferative capacity ex vivo, together with attenuated muscle regeneration after cardiotoxin injury in HFD muscle [160].

In humans, myoblasts derived from patients with metabolic dysfunction also retain intrinsic defects. Metabolic studies show that myotubes derived from patients with T2D retain a diabetic phenotype in culture, including increased lactate production, decreased glucose uptake as well as incorporation of glucose into glycogen, and increased expression of genes involved in mitochondrial and fatty acid metabolism [161,162]. A separate investigation showed increased pyruvate dehydrogenase kinase 4 expression after stimulation with fatty acids (palmitic or oleic acid) in T2D myotubes [163]. Furthermore, myotubes from extremely obese patients secrete 2.9-fold more myostatin, an inhibitor of skeletal muscle hypertrophy, compared with lean controls [164]. The effect of caloric restriction on MuSCs is considerably more difficult to assess in humans, but evidence suggests that, as in mouse models, it may have a beneficial effect on muscle metabolism in humans [165,166].

SATELLITE CELL SELF-RENEWAL MECHANISMS

The MuSC pool is maintained by self-renewal, which results from two proliferation mechanisms: (1) asymmetric self-renewal, in which a stem cell divides into one differentiated cell and one stem cell; and (2) symmetric self-renewal, in which a stem cell divides to become two equivalent stem cells. MuSCs can also remain quiescent or

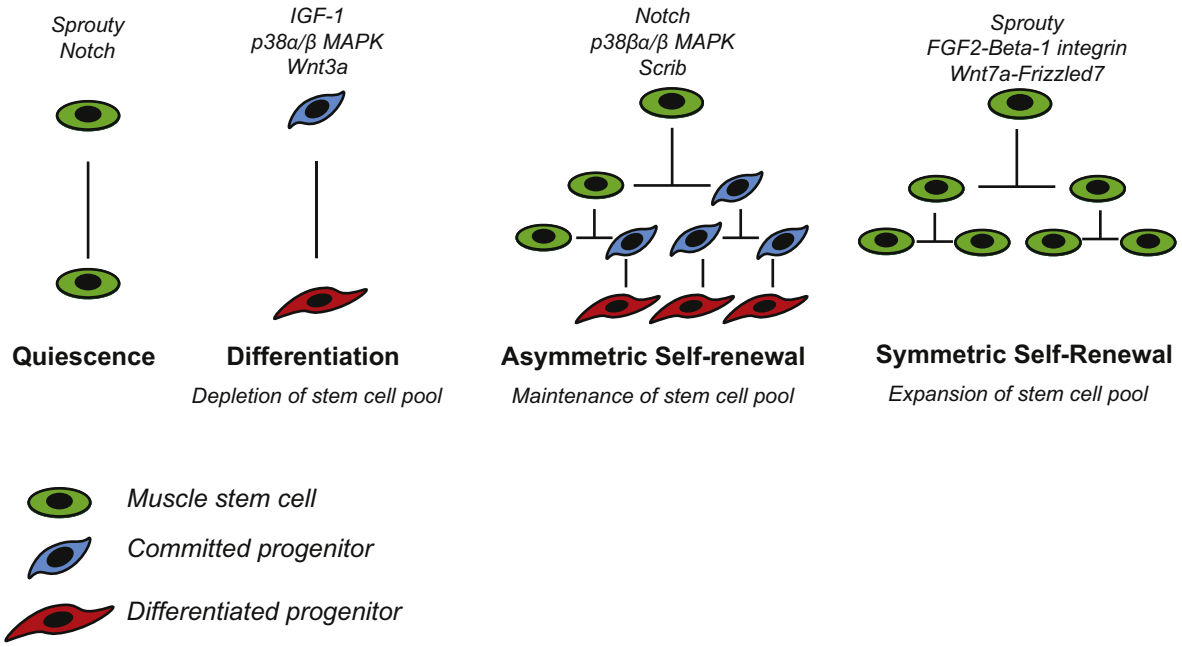

FIGURE 18.3 **Muscle stem cell (MuSC) fates during regeneration and promoting factors.** During regeneration, the quiescent MuSC pool can be depleted through death and differentiation. After activation by injury, this pool can be maintained through two types of self-renewal: asymmetric and symmetric. In asymmetric stem cell self-renewal, a stem cell divides into one committed progenitor that will eventually differentiate and one stem cell maintains the MuSCs pool. Expansion of the MuSC pool occurs with symmetric stem cell self-renewal when a stem cell divides to generate two equivalent stem cells. *FGF2*, fibroblast growth factor-2; *IGF-1*, insulin-like growth factor-1; *MAPK*, mitogen-activated protein kinase.

differentiate (Fig. 18.3). Under homeostasis, it is believed that asymmetric self-renewal is sufficient to meet the demands of normal cell turnover. However, during injury the demand for expansion of stem cells likely also requires symmetric self-renewal [167]. In the mouse, self-renewal mechanisms of MuSCs have been studied through lineage tracing with the transgenic *Myf5-Cre/ROSA26-YFP* mouse [21,34], which expresses yellow fluorescent protein (YFP) after expression of the MuSC activation gene *Myf5*. These studies showed that Notch signaling drives the asymmetric division of quiescent $PAX7^{+}MYF5^{-}$ MuSCs into one $PAX7^{+}MYF5^{-}$ and $PAX7^{+}/MYF5^{+}$ cell [34], and that WNT7A signaling drives the symmetric division of one $PAX7^{+}/MYF5^{-}$ MuSC into two $PAX7^{+}/MYF5^{-}$ cells [21]. Differences between symmetric and asymmetric division have also been demonstrated through dye-dilution studies, which identified slow-dividing MuSCs capable of generating progeny with long-term self-renewal properties [168,169]. Similarly, bromodeoxyuridine labeling of transgenic *Pax7-nGFP* mice showed that MuSCs that express high levels of *Pax7* ($Pax7^{Hi}$) have higher self-renewal capacity compared with MuSCs with low levels of *Pax7* ($Pax7^{Lo}$). Furthermore, in accordance to the immortal strand hypothesis, $Pax7^{Hi}$ MuSCs undergo asymmetric chromosome segregation, allowing the template DNA strand to segregate exclusively to one daughter cell to maintain its stem cell identity. In contrast, $Pax7^{Lo}$ MuSCs show random chromosome segregation [35].

The polarity of signaling components in the stem cell niche dictates whether MuSC division is asymmetric and symmetric. Apical-basal polarity cues orient the mitotic spindle during division, leading to asymmetric stem cell division perpendicular to the basal lamina [34,170], whereas symmetric divisions occur in a planar fashion [21,105]. During asymmetric division, quiescent $PAX7^{+}/MYF5^{-}$ MuSCs result in one $PAX7^{+}/MYF5^{-}$ and one $PAX7^{+}/MYF5^{+}$ cell. In most of these divisions, the $PAX7^{+}/MYF5^{+}$ cell is pushed apically toward the host myofiber whereas the daughter cell that remains on the basal lamina is $MYF5^{-}$ [34]. The Notch ligand *Delta1* is upregulated in the $PAX7^{+}/MYF5^{+}$ daughter cell. Pharmacological inhibition of Notch signaling decreased cell division and increased the number of differentiated $PAX7^{-}/MYOD^{+}$ cells, which implicates the Notch pathway in the maintenance of self-renewal through asymmetric divisions as well as quiescence [134]. It has also been shown that dystrophin, which is primarily expressed by the muscle fiber, also regulates the polarity of MuSC division through microtubule affinity regulating kinase 2 [170]. Dystrophin is expressed when MuSCs are activated and is localized asymmetrically to one side of the cell just before division. In the absence of dystrophin, asymmetric division is impaired, leading to the loss of apicobasal division and a reduction in the number of myogenic progenitors.

Evidence has shown that polarity may also be directed by the basolateral Scribble complex, which is expressed in MuSCs upon activation. During asymmetric division, it becomes segregated into the progenitor cell committed to differentiation. It can also segregate symmetrically when both cells differentiate [171].

Symmetric divisions result in two equivalent daughter cells and occur in a planar cell orientation. WNT7A is secreted by regenerating myofibers and directs symmetric cell division through noncanonical Wnt signaling mediated through the FZD7 receptor to stimulate the planar cell polarity pathway (PCP). Stimulation of MuSCs on isolated myofibers with WNT7a induces symmetric cell division and polarized localization of the PCP protein VANGL2 on opposite ends of daughter cells [21]. In addition, WNT7A increases motility and migration of MuSCs through the Rho-GTPase Rac1 [139]. Upstream, FZD7 is part of a coreceptor complex with SDC4, which binds to the ECM protein fibronectin [105]. Fibronectin is produced by myogenic progenitors and fibroblasts in the regenerating muscle. Transient upregulation of fibronectin enhances WNT7A-mediated symmetric expansion and inhibits differentiation, whereas loss of fibronectin impairs maintenance of the MuSC pool. STAT3 signaling has been shown to be involved in maintenance of quiescence; studies showed that acute inhibition leads to MuSC expansion, increased symmetric cell divisions, and improved muscle regeneration [172,173]. However, other studies showed that chronic, long-term ablation of *Stat3* leads to gradual depletion of MuSCs and the loss of regenerative capacity over time [174].

An alternative potential mechanism of satellite cell self-renewal is reversion from a committed myogenic progenitor back into a quiescent satellite cell. In mice, it was observed that a rare subset of myofiber-derived $PAX7^{+}MYOD^{+}$ progenitors do not differentiate further into myogenin^{+} cells but instead lose *MyoD1* expression and revert to a quiescent $PAX7^{+}/MYOD^{-}$ satellite cell state [175]. This reversion may be mediated by the upregulation of SPRY1, a negative regulator of growth factor–activated receptor tyrosine kinase signaling that is associated with the reestablishment of satellite cell quiescence after muscle regeneration [128]. These reports suggest that maintenance and expansion of the satellite cell pool are governed by self-renewal mechanisms that, although incompletely understood, are likely regulated by the active presentation of specific niche-associated extrinsic signals in a dose-dependent, temporally, and spatially controlled manner [139]. Ex vivo, inhibition of the tumor suppressors pRb as well as p19ARF has been shown to be sufficient to reverse committed postmitotic myocytes to the proliferative progenitor state [176]. This reversion process, known as dedifferentiation, has been shown to involve a mechanism characteristic of early programmed cell death [177]. Future studies that replicate the niche with bioengineered platforms or interfere with niche signals will facilitate maintenance and expansion ex vivo for subsequent transplantation; studies that target and activate the endogenous activation of MuSCs will find useful applications in regenerative medicine.

MUSCLE STEM CELL–INTRINSIC DEFECTS IN AGING AND DISEASE

With aging and disease, the stem cell capacity of MuSCs declines both intrinsically and extrinsically. Cellular turnover as well as extrinsic changes to the environment result in intrinsic alterations to MuSC fate, leading to compromised muscle mass and function. The number of $PAX7^{+}$ MuSCs per myofiber decreases with age, concomitant with a decline in regenerative capacity and muscle mass [119]. Even before MuSC numbers decline appreciably (>18 months in the mouse) [137,178], however, there is a shift toward greater heterogeneity in the MuSC population. With age, there is an increased proportion of MuSCs with reduced clonal proliferation capacity and a smaller fraction is able to develop into muscle myofibers when transplanted into recipient muscles [26]. Aged MuSCs exhibit increased expression of the senescence marker $p16^{Ink4a}$, elevated phosphorylation of the inflammatory p38α/β MAPK pathway, and upregulation of inflammatory cytokines and cytokine receptors [26,123,137,179]. These age-associated changes in MuSCs accompany sarcopenia, or age-related muscle wasting. During sarcopenia there is a reduction in the number of nuclei leading to myofiber atrophy [7]. Evidence showed that MuSCs contribute to myofibers even in the absence of injury [11]. There is also evidence suggesting that inducible depletion of MuSCs slightly worsens sarcopenia [11], although other studies claimed it is insufficient [8].

During aging there is also an accumulation of senescent cells that are alive but nondividing. These cells produce an array of cytokines, growth factors, and proteases, known as the senescence-associated secretory phenotype (SASP) [180], which has been shown to be beneficial for wound repair [181]. SASPs have also been causally implicated in generating age-related phenotypes in adipose tissue, skeletal muscle, and the eye [182]. The senescent marker $p16^{Ink4a}$ is upregulated in geriatric (age ~75 years) human satellite cells compared with young (age 25 years) counterparts and is reduced in myogenesis [179]. In geriatric mice (age >24 months), $p16^{Ink4a}$ expression leads to reduced activation after injury and accumulation of senescent MuSCs, resulting in impaired muscle regeneration. Conditional deletion of the $p16^{Ink4a}$ gene in $PAX7^{+}$ MuSCs restores the capacity of quiescent geriatric MuSCs to be activated and reduces the expression of senescence-associated genes.

In addition to senescence, a number of signaling pathways are dysregulated in aging. Many are pathways that are transiently upregulated or downregulated during activation in young, healthy MuSCs after injury. For example, the balance of Notch and Wnt pathways is critical during regeneration, with a temporal switch from Notch to Wnt during normal myogenesis [138]. Notch signaling is important for MuSC activation and self-renewal, whereas Wnt antagonizes Notch and promotes differentiation. However, during aging, expression of the Notch ligand Delta-1 decreases in muscle. Consequently, MuSCs are exposed to increased levels of WNT3A (wingless-type mouse mammary tumor virus integration site family, member 3A), which impair self-renewal and induce fibrogenic differentiation of MuSCs and muscle progenitors through β-catenin signaling [137]. Oxytocin is another signaling molecule that is altered in aged mice. Better known for its role in lactation and social behaviors, oxytocin is expressed systemically, and in young MuSCs it promotes muscle regeneration. Expression of oxytocin declines in aging. Depletion of oxytocin in young mice causes premature sarcopenia, whereas repletion results in proliferation and rejuvenates MuSC function [183].

Chronic inflammation and dysregulation of inflammatory pathways are particularly potent drivers of aging phenotypes in MuSCs. A temporally regulated, transient immune response is a critical component of regeneration. However, aging leads to chronic inflammation and elevation of cytokines, resulting in aberrant cell-autonomous activation of the stress-associated p38α/β mitogen-activated protein kinase (MAPK) and the cytokine-stimulated JAK2/STAT3 signaling axes, among others [26]. Increased activity of p38α/β suppresses *Pax7* expression through repressive chromatin modifications [184] and results in the loss of self-renewal capacity [26,185]. Inhibition of p38α/β activity has been shown to rejuvenate stem cell function of aged MuSCs [26]. Activation of JAK2 leads to activation of its target STAT3, which causes asymmetric division and *MyoD1* expression in MuSCs [172,173]. In vitro and transplantation experiments in vivo showed that acute inhibition of JAK phosphorylation or STAT3 activity promotes symmetric MuSC expansion and rescues muscle regeneration in aged and dystrophic mice [172,173]. Factors secreted by muscle fibers during regeneration are also dysregulated in aging. Aged muscle fibers aberrantly express the growth factor *Fgf2* in homeostasis, which causes a subset of MuSCs to exit quiescence and lose their self-renewing capacity owing, in part, to reduced SPRY1 levels [123]. Normal MuSCs from healthy young animals typically express SPRY1, which inhibits FGF signaling in vivo.

MuSCs isolated from diseased muscle have compromised regenerative capacity and stem cell function. In the *mdx* mouse model of DMD, which lacks the key muscle structural protein dystrophin, MuSCs in limb and diaphragm muscles are forced to undergo constant rounds of activation and proliferation because of the constitutive degeneration of the tissue. Early studies showed that myoblasts isolated from patients with DMD exhibit a reduction in proliferative capacity and senesce more readily than myoblasts isolated from healthy controls [18,186,187]. Reduced proliferation and self-renewal capacity are also observed in MuSCs from the *mdx/mTR*^{G2} humanized model of DMD [20]. To generate this mouse model, which has shortened telomeres with lengths approximating those of humans, *mdx* mice were crossed with the *mTR* mouse, which lacks telomerase activity because of the absence of the RNA component of the enzyme. MuSCs from these animals have reduced proliferative capacity after injury as well as a reduced capacity to engraft when transplanted into recipient WT muscle. This cell-intrinsic restriction of self-renewal and proliferation further revealed disease phenotypes in the mouse that are present in human patients, but were not previously observed in the *mdx* mouse alone: namely, progressive muscle degeneration across skeletal muscle types and premature death resulting from dilated cardiomyopathy [20,188]. The role of MuSCs in other genetic myopathies remains to seem.

CHALLENGES IN THE USE OF SATELLITE CELLS IN REGENERATIVE MEDICINE

MuSCs have a great potential to be used a cell therapy; however, many significant challenges remain unmet for the use of human satellite cells in transplant-based clinical regenerative medicine applications. Although much progress has been made toward identifying surface markers to isolate human MuSCs prospectively, satellite cell transplantations require intramuscular injections to deliver functional stem cells efficiently to recipient muscle tissue. This cell type is unable to access muscle tissue when delivered systemically through the vasculature [189,190]. Therefore, treating all muscles of the body is untenable. This limitation restricts the use of MuSC transplantations to small and easily accessible muscles and makes regenerative therapy impractical for all muscles or muscles that are difficult to access, such as the diaphragm.

Before the identification of satellite cell isolation markers, researchers and clinicians long proposed using human myoblasts as a source of cells for muscle therapeutic applications. Myoblasts can be generated from enzymatically digested muscle tissue or isolated satellite cells [191] and rapidly proliferate in culture. Because they can be

maintained and expanded in culture while retaining myogenic potential, myoblasts are an attractive source for cell-based therapies. In clinical trials in the 1980s, transplanted human myoblasts were shown to fuse with endogenous muscle fibers and led to the production of functional dystrophin in the myofibers of patients with DMD [85,192]. However, a large proportion of myoblasts die after injection, expansion is extremely limited, and they do not engraft into a stem cell niche and thus ultimately fail to provide long-term therapeutic benefit.

These clinical findings are corroborated by engraftment studies of satellite cells and myoblasts transplanted into damaged mouse muscles. A major challenge for preclinical and clinical studies is that MuSCs in mice and humans are rare (about 5% of all muscle nuclei), and cannot be maintained for more than a few days. Therefore, expansion ex vivo is limited. Culture of satellite cells for more than a few days in standard tissue culture conditions results in conversion to a myoblast phenotype with a dramatically diminished self-renewal potential [30,76]. Improvements have been made in culture platforms, which have allowed for the extended maintenance of MuSC potential in vitro. Biomimetic materials have been developed that can be precisely fine-tuned to mimic biophysical cues provided by the MuSC niche. Engineered poly(ethylene glycol) (PEG)-based hydrogels have been engineered that replicate the physiological stiffness of the MuSC environment [149], which is five orders of magnitude less stiff than tissue culture plastic, allowing maintenance of stem cell potential. These hydrogels can be functionalized with specific tethered ligands, including adhesion and growth factors, and are resistant to degradation and nonspecific protein absorption [193]. Hydrogels are advantageous over Matrigel, which is composed of uncharacterized amino acids and growth factors. Artificial muscle myofiber preparations have also been developed to mimic the stem cell niche of the myofiber, using collagen-based hydrogels to fine-tune the stiffness of the substrate [194]. Like PEG-based hydrogels, these artificial myofibers promote stemness combined with defined media conditions, leading to enhanced engraftment after transplantation. In addition to biophysical cues, cell culture media have been optimized. Screens have identified a number of defined factors, including forskolin, p38/MAPK inhibitors, and TGF-β, which promote MuSC expansion and self-renewal [26,194,195]. A synergy of biophysical and biochemical cues provided by 12-kPa hydrogels and the p38/MAPK inhibitor SB202190 rejuvenates the function of the aged MuSC populations. After transplantation, aged mouse muscles exhibit strength on par with young ones. Nonetheless, further experimentation is warranted to establish methods to extend the culture life span and propagation of MuSCs for cell therapeutic application. The need is even greater for satellite cells bearing genetic defects, such as those present in patients with DMD, that require ex vivo gene editing before transplantation.

GENE EDITING STRATEGIES

Advances in gene editing strategies have created the possibility of autologous cell therapy transplantation for patients with genetic disorders. Through gene editing, genetic defects such as dystrophin mutations found in DMD can potentially be corrected through systemic delivery, or in MuSCs isolated from patients, which would then be transplanted back into the tissue. A number of strategies have targeted muscle systemically, including viral delivery of mini- or microdystrophin genes by adenoassociated virus (AAV) vectors [196,197], and oligonucleotide mediated exon skipping to restore the dystrophin reading frame [198,199]. Notable progress has been made using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated-9 (Cas9) genome editing system, which targets DNA sequences with a single guide RNA and cleaves the target with the Cas9 nuclease [200]. Proof-of-concept studies have demonstrated the feasibility of AAV-mediated delivery of CRISPR/Cas9 to the skeletal muscle of the dystrophic *mdx* mouse model [201–203], partially restoring dystrophin expression and skeletal muscle function. Restoration of dystrophin was primarily shown for myofibers. However, these strategies still must be optimized to increase efficiency. Moreover, targeting MuSCs, which would lead to long-term correction, remains a challenge, and subsequent gene editing in a nondividing cell constitutes an additional hurdle. Also, specific CRISPR/Cas9 correction requires targeting sequences to be tailored for each patient's mutation. Regardless, progress is rapidly being made, offering hope for tenable gene therapy options.

OTHER STEM CELL TYPES WITHIN MUSCLE

Several other myogenic cell types have been proposed as alternatives to the satellite cell. A number of other alternative MuSC sources and muscle-resident nonmuscle cells that contribute to regeneration have been characterized.

These include blood vessel–associated mesoangioblasts [204,205], pericytes [189,206], and myogenic cells derived from induced pluripotent stem (iPS) cells [207]. These cell types are discussed in this section. Although many of these cell sources have potential characteristics for cell therapy applications, their roles in normal development and muscle regeneration and their anatomical locations have not been fully documented. They may not represent the native MuSC but they may have therapeutic utility.

Other Stem Cell Types Within Muscle

A number of other cell types in the muscle are integral to regeneration. Muscle-resident cell types including blood vessel–associated mesoangioblasts, pericytes, FAPs, and TWIST2$^+$ (Tw2$^+$) myogenic progenitors have been shown to be contribute to regeneration in addition to MuSCs. Mesoangioblasts are blood vessel–associated multipotent mesodermal progenitors that can be isolated from fetal muscle biopsy tissue fragments containing small blood vessels [205]. They are an attractive source because they can be expanded in culture and contribute to muscle regeneration effectively throughout the body without tumor formation after intraarterial delivery owing to their ability to extravasate from the vasculature. Reliance on fetal tissue to isolate mesoangioblasts can be overcome by using an alternative but related population of blood vessel–associated cells called pericytes that are present in adult humans [189]. The advantageous characteristics of mesoangioblasts have motivated their extensive preclinical evaluation, including in the Golden Retriever muscular dystrophy model, which closely mimics human DMD [204]. However, clinical trials have shown that although mesangioblast transplantation is relatively safe, it has limited efficacy in human patients [208].

Fibroblasts and adipocytes within the muscle tissue arise from FAPs, also characterized as nonmyogenic mesenchymal stem cells. FAPs can be isolated based on CD45$^-$/CD31$^-$, α7-integrin$^-$, SCA1$^+$, and CD34$^+$ cell-surface marker expression [209]. Mesenchymal stem cells can be isolated based on CD45$^-$/CD31$^-$, SM/C2.6$^-$, and PDGFRα^+ expression, parameters that select for a functionally and phenotypically equivalent population of cells to FAPs [210]. Both cell types are capable of differentiating into adipocytes and fibroblasts in vitro, producing both α-smooth muscle actin–expressing fibroblasts and perilipin/peroxisome proliferator-activated receptor γ–expressing adipocytes. Upon injection of glycerol, transplanted FAPs and mesenchymal progenitors underwent adipogenic differentiation in vivo. Although fibrosis is generally associated with aging and disease, muscle-resident fibroblasts are necessary for regeneration [211]; ablation leads to decreased myofiber size and premature MuSC differentiation. Reciprocally, during regeneration, muscle progenitors have been shown to regulate ECM deposition by fibrogenic cells within the muscle through secreted exosomes containing MiR-206 [212].

Finally, the transcription factor Tw2 was identified as a marker of a separate population of interstitial progenitor cells [212,213]. Tw2$^+$ progenitors are distinct from PAX7$^+$ MuSCs. When isolated, they can enter the myogenic pathway by activating PAX7 and MYOD1. In vivo, Tw2$^+$ progenitors contribute specifically to type IIB/X fibers during homeostasis and are required for maintenance of type-IIB/X fiber size. However, Tw2$^+$ progenitors cannot sustain regeneration in the absence of PAX7$^+$ MuSCs.

Induced Pluripotent Stem–Derived Muscle Stem Cells

IPS cell–derived MuSCs have been developed as a tool to study human myogenesis in regeneration and disease as well as to provide a source for cell therapy–based therapies. Human and mouse muscle cells have been derived from embryonic stem cells or iPS cells through overexpression of myogenic transcription factors such as MYOD1, PAX3, or PAX7 [207,214–217]. Studies have demonstrated the generation of human and mouse muscle progenitors capable of differentiating into mature myofibers using exogenous signaling factors [218–220] These protocols were derived from an understanding of developmental principles and sequentially expressed regulators. As such, they all share similar features of activating Wnt and inhibiting Notch signaling, a protocol that has also been used to derive skeletal muscle progenitors and myocytes from human pluripotent stem cells [221]. A major advantage of human iPS cells is that they constitute an unlimited cell source that can be infinitely propagated. A major drawback is that derivation of human iPS cells and subsequent differentiation into PAX7$^+$ MuSCs is lengthy and inefficient. Optimization is needed, but much progress has been made, and now that markers of adult human MuSCs have been characterized, flow cytometry enrichment protocols are at hand. As such, human IPS cells hold great promise for muscle cell therapies in the future.

CONCLUSIONS

Muscle satellite cells are essential for postnatal muscle regeneration and maintenance. They have been shown to be native MuSCs, and their study has provided critical insights into the mechanisms of muscle regeneration. However, their therapeutic use continues to be restricted by cell transplantation constraints, including the inability to contribute to myogenesis extensively when delivered systemically, and their limited capacity to be expanded and maintained in culture. Technological advances such as improved cell culture platforms and iPS cell−derived human MuSC strategies offer great promise to overcome these limitations. In addition to improving satellite cell transplantation in preclinical models, advances in MuSC in vitro technologies have led to the identification of SB202190 and should prove useful for identifying additional therapeutic agents that stimulate the regenerative contributions of satellite cells. Efforts to augment the function of endogenous satellite cells within muscle tissues are warranted for muscle wasting caused by sarcopenia or cachexia, but not conditions such as muscular dystrophy, for which such agents would exacerbate the disease. Furthermore, novel noninvasive imaging methods such as BLI and IVM enable the quantitative assessment of regeneration over time. Advances in isolating human MuSCs, iPS technology, and the validation of alternative cell types capable of contributing to human muscle regeneration may lead to the clinical realization of much-needed stem cell therapies for prevalent muscular dystrophies and muscle wasting conditions that accompany aging.

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CHAPTER

19

Stem Cells Derived From Fat

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INTRODUCTION

Regenerative medicine aspires to restore the physiologic function of a target tissue by way of reconstitution and/or reproduction using a combination of medicine, engineering, and molecular biology. Emerging strategies require the use of multipotent cells, and given the controversies surrounding embryonic stem cells, adult stem cells (particularly those derived from autologous sources) have been targeted by scientists and physician alike to achieve regeneration. In the late 1980s, Caplan et al. coined the term "mesenchymal stem cell" (MSC). This name was chosen in light of the inducible differentiation of these cells into osteogenic, chondrogenic, and adipogenic lineages [1].

A growing body of evidence indicates that many different adult tissues contain MSCs, including bone marrow (BM), blood, nervous tissue, skeletal muscle, and fat. Because of their almost ubiquitous presence and because several studies microanatomically localized MSCs adjacent to blood vessels, many independent groups have proposed a "unified theory": MSCs are resident in the perivascular niche. Other groups have advanced this theory further: MSCs are *not resident in* but *originate from* a heterogeneous group of perivascular cells, namely pericytes ($CD45^-/31^-/34^-/146^+$) and adventitial cells ($CD45^-/31^-/34^+/146^-$) [2–5]. Histologically, pericytes are in close proximity to capillary endothelium whereas adventitial cells are resident some distance from the largest vessels in the tunica adventitia, far from endothelial cells. The cluster of differentiation profiles used to describe these populations has been classically defined using flow cytometric studies of culture-expanded populations. Because of the ability of adventitial cells to assume pericyte phenotypes in vitro, these phenotypes represent a multipotent cell population that, if tapped, may alter the trajectory of cell-based therapies and regenerative science.

Adipose tissue is unique as a source of MSCs. Historically, fat was relegated to energy storage and structural supportive roles. However, enzymatic dissociation and centrifugation of adipose yielding a single-cell suspension, called the stromal vascular fraction (SVF), allowed the characterization of its cellular components, including the adipose-specific MSC: the adipose-derived stromal cell (ASC) [6]. One gram of adipose tissue obtained with minimally invasive liposuction yields approximately 5000 putative stem cells, which is 500 times greater than the number of stem cells isolated from 1 g of BM [7]. In addition, approximately 400,000 liposuction surgeries are performed in the United States each year, routinely yielding volumes of up to 3 L of valuable tissue [8]. Thus, adipose offers autologous mesenchymal progenitors with multilineage differentiation potential in unrivaled quantity, accessibility, and expendability.

CELLULAR FRACTIONS

In 1964, Martin Rodbell described the first enzymatic dissociation of murine adipose tissue into a single-cell suspension. Since then, many studies have demonstrated reproducible collagenase digesting and isolating cellular fractions either intact (and subsequently minced) or aspirated with tumescent liposuction. After enzymatic neutralization, which is often achieved by albumin and/or volumetric dilution, lipid-laden, mature adipocytes are separated from remaining cellular fraction using differential centrifugation. The resulting cellular fraction, which is devoid of mature fat cells, is a primary, heterogeneous, mesenchymal cell array collectively termed the SVF. The uncultured SVF consists of leukocytes, erythrocytes, endothelial cells, perivascular cells, and of particular interest, putative stem and progenitor

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cells. When the heterogeneous SVF cells are cultured in plastic, spindle-shaped cells attach and expand. These cells, selected by plastic adherence, are ASCs.

As the field of adipose science stands, the overwhelming majority of work has involved the biological evaluation and therapeutic projection of ASCs. Given the inherent heterogeneity of the SVF, there is a paucity of studies comparing the efficacy (and its dependent potency) of SVF in culture-expanded ASCs. Perhaps more important, the literature is unintentionally pervaded by the inaccurate transposition of SVF/ASC terminology. The cellular characterization of both populations has been a subject of scientific interest, as evidenced by even a cursory literature search, likely caused by acceleration in the methodology and technology used to isolate adipose-derived cell populations. To ensure the integrity of interstudy comparisons and subsequent scientific advancement within the field of adipose, a consensus biological profile of each adipose-derived cell population is required and can provide insight into biologic clarifications, best clinical practices, and safety profiles [9]. More important, this consensus profile must be dynamic and responsive to the newly acquired data and resulting advancements within adipose science, so as not to strangle the field at this juncture of critical growth.

CELLULAR CHARACTERIZATION

Stromal Vascular Fraction

The SVF, which is marked by its heterogeneity, is composed not only of perivascular cells, leukocytes, and endothelial cells, but also of a higher percentage of stromal cells compared with the BM nucleated fraction (which contains BM-MSCs). The SVF is composed of less than 0.1% of stem and progenitor cells. These cells of traditional interest (the stromal population) have a cluster of differentiation profile that includes positivity for CD34 and negativity for CD45 (leukocyte common antigen), CD235a (glycophorin A), or CD31 (platelet-endothelial cell adhesion molecule-1 [PECAM-1]); therefore, its immunophenotypic profile in common notation should be $CD45^{-}CD235a^{-}CD31^{-}CD34^{+}$. CD34, likely because of variation in the epitope of which class I/II/III-CD34 antibody detects, is positive with variable intensity [10]; for SVF cell characterization, Bourin et al. recommends the use of class III CD34 antibodies to obtain the most consistent signal [9]. In addition, the following surface antigens may be helpful in identifying the stromal population, particularly combined with the CD profile: CD13 (aminopeptidase N), CD73 (lymphocyte-vascular adhesion molecule-2), CD90 (Thy-1), and CD105 (endoglin).

Adipose-Derived Stromal Cell

The definitive cell surface identity of ASCs has historically been problematic. Surface protein expression is dynamic, partially depending on the passage and cell density, among other variables including the anatomic site of harvest, isolation methodology, and culture protocol. However, after two or more passages, ASCs express relatively characteristic and reproducible surface and adhesion molecules, cytoskeletal elements, and surface markers. The ASC immunophenotype is comparable to that of the SVF stromal population, with a lack of CD45, CD235a, and CD31 expression. With regard to surface antigens, however, CD13, CD29 (integrin β1), CD44 (homing cell adhesion molecule), CD73, CD90, and CD105 are the primary, consistently stable ASC markers. Overall, the ASC immunophenotype largely resembles that reported for other adult MSCs, such as BM-derived and skeletal muscle stem cells, with only a few differences [6,11–13]. However, in conjunction with selection by adherence to plastic, these immunophenotypic differences suggest that ASCs are indeed a distinct population. In light of this, several studies have hypothesized and confirmed preferential differentiation programs of ASCs versus BM-MSCs; ASCs demonstrate a greater tendency to develop into myocytes, and BM-MSCs into osteocytes and chondrocytes [14].

Ultimately, cell populations are best characterized by biological activity, multipotency, and differentiation program [6,13,15] ACSs have been shown to demonstrate phenotypes consistent with several different mesodermally, endodermally, and ectodermally derived mature cell types both in vitro and in vivo. This has been reflected in the adipose scientific industry; a prime example is the broad selection of lineage-specific inductive media and associated protocols. Although these developmental findings are remarkable and continue to evolve, many questions remain as well as some appropriate residual skepticism related to the "applied" in vivo developmental plasticity and functional integration of adult stem cells. Part of this relates to limitations of terminology, standardization, and existing technologies, and also to the human need to characterize the elusive nature of dynamic living systems with static labels and/or pathways. For example, establishing a scientific consensus regarding which "minimal threshold" definitively describes a particular phenotype/cell lineage is elusive, to say the least, and the "minimal essential threshold" continues to evolve as the field matures and realizes that there are few if any genes or proteins that

are truly "lineage specific." These issues are greatly interesting and important for basic scientists and developmental biologists, but the ultimate benchmark of these cells for many in the fields remains the reproducible and predictable therapeutic safety and efficacy in the clinical setting, regardless of the mechanism of action.

Cell Source

All fat is not the same. There are two main types of fat within the human body: brown adipose tissue (BAT) and white adipose tissue (WAT). BAT is mainly found surrounding the viscera in infants; in adults it has been identified in the neck, supraclavicular, paraaortic, paravertebral, and suprarenal areas [16]. BAT serves primarily to generate heat via a specific uncoupling mechanism identified in the cells' mitochondrial electron transport chain. WAT is found subcutaneously, is highly vascularized, and contains the ASC population; its primary function is to serves an energy reserve, but it is recognized as a dynamic and highly bioactive tissue with important roles in systemic inflammation and other processes [3,17]. Major depots of subcutaneous WAT in humans include abdominal, hip, thigh, and gluteal locations.

Population ratios of adipose-derived fractions vary depending on the anatomic site from which fat is harvested, as well as donor characteristics such as age, sex, smoking history, and medical comorbidities. In addition, studies indicate that the density of ASCs varies depending on many of these factors [18,19]; these may be of particular importance when considering ASC yield, plasticity, and potency.

CLINICAL DELIVERY OF ADIPOSE-DERIVED CELLS

Several potential ASC delivery routes exist, including systemic, parenteral, targeted injection, and topical application.

The systemic administration of ASCs as a cell suspension continues to have great translational appeal because it provides a minimally invasive treatment modality. Imperative to this delivery modality is the characteristic of ASCs of mobilizing to injured tissue, termed homing. However, the mechanism of homing is poorly understood in ASCs and other MSCs. Scientific insight into homing can be found in inflammation, which is composed of three major stages: the dilation of capillary beds and subsequent increase in blood flow; protein expression and microvascular structural changes; and the leukocyte adhesion cascade involving white cell capture, adhesion, and transmigration. Despite the native inability to bind endothelium via E-selectin, Pachon-Pena et al. described the differential expression of sialyllactosamines (i.e., oligosaccharide residues) on glycovariant cluster of differentiation markers, specifically CD44 [20,21]. These glycovariants can subsequently be fucosylated, creating hematopoietic cell E-/L-selectin ligand, a membrane constituent that permits the shear-resistant engagement of human adipose-derived MSCs to endothelium. Thus, differential protein expression and selective posttranslation modification may allow MSCs to bind selectively to sites of inflammation.

In addition, the leukocyte adhesion cascade is highly dependent on injured tissues' differential, attractant expression of chemotactic cytokines (chemokines), and leukocyte chemokine receptors. In fact, the chemokine receptor expression profiles of leukocytes and ASCs have been demonstrated to overlap significantly, including those with assigned functional roles of transendothelial migration, diapedesis, and vascular permeability (e.g., CCR1, CCR7, CXCR3, CXCR4, and CXCR6) [22–26]. Therefore, the "native" homing mechanism of ASCs may be similar to that of leukocytes, composed of differential chemokine receptor expression or differential glycoprotein expression first-pass removal through "filter organs," or the result of postinjury, increased perfusion in a damaged organ, or most likely, a combination of these.

With parenteral delivery, ASCs engraft into many organs and concentrate largely in first-pass organs (lung and liver) as well as the spleen. Several studies have indicated additional engraftment sites for MSCs (a group to which ASCs belong) including thymus, brain, spinal cord, and heart. There has been evidence that there is variability in the chemokine receptor expression profiles of ASCs [27,28]. This may indicate that there are phenotypic and induced protein expression differences to modifiable culture conditions (e.g., supplements, pH, cell density, and/or matrix). This variability may also confirm that ASCs have the ability to home to multiple tissues, as was previously demonstrated. Therefore, modulation of chemokine receptor expression has become an area of growing interest to increase the engraftment efficiency of cells to a specific, often injured target tissue. In 2016, Hasemzadeh et al. demonstrated that differential expression could be primed with valproic acid, which resulted in increased CXC chemokine expression without altering CC chemokine groups [29]. In turn, this may influence their migration and engraftment

patterns and improve the efficacy of ASC cell therapies while decreasing the cell dose and minimizing the side effect and morbidity profiles [30–32].

It has been reported that ASCs possess the ability to differentiate into cardiomyocytes, both in vitro and in vivo, with an intact electrolyte-dependent, spontaneous, rhythmic contraction ability [33–36]. Guided by this potential, a large portion of research involving the parenteral delivery of ASCs has been aimed at cardiovascular disease, with the goal of mitigating ischemic damage and/or regeneration of myocardium. There have been several articles reporting positive effects to this end, which have been attributed to improved vascular inflow, increased angiogenesis, and cell protection (i.e., reduction in apoptosis) [37,38]. In replications of studies using marrow-derived MSCs, independent groups demonstrated that ASCs differentiate into endothelial and vascular smooth muscle cells when injected in the coronary artery supplying acutely infarcted porcine myocardium, limiting infarct size, thickening the left ventricular wall, and improving cardiac function [39,40]. In 2011, intravenous administration at a distant site by Kim et al. demonstrated homing of ASCs to, and cardiomyocyte differentiation in, canine radiofrequency-ablated atrium [41]. Some studies indicated ASC engraftment into infarcted myocardium without adopting the cardiomyocyte morphology or phenotype; it is hypothesized that paracrine effects of ASCs assisted in remodeling because of improved cardiac wall architecture [42]. Two prospective, double-blind, randomized, controlled, phase I human clinical studies using ASCs were undertaken, both initiated in 2007: Adipose-Derived Stem Cells in the Treatment of Patients With ST-Elevation Myocardial Infarction (APOLLO) and Adipose-Derived Stem and Regenerative Cells in the Treatment of Patients With Nonrevascularizable Ischemic Myocardium (PRECISE). In contrast to most preclinical literature, both trials used the heterogeneous, autogenous SVF as opposed to culture-expanded ASCs. There is scientific thought that the heterogeneity of the SVF may offer measurable benefits if this heterogeneity can be recapitulated in the injected or implanted graft [23,43,44]. The APOLLO and PRECISE studies were completed with an estimated enrollment of greater than 80 patients, but preliminary experimental results for the former are not available [45].

Similarly, ASCs have demonstrated therapeutic potential in peripheral vascular disease. Intravenous administration of murine ASCs attenuates critical limb ischemia to lower limbs [46,47]. The ability of ASCs to alter the extracellular, ischemic micromilieu is not brought about solely by directed target tissue differentiation, but likely involves the adipose secretome, the entirety of ASC-secreted cellular products. Several independent groups have demonstrated ischemic recovery caused by the paracrine secretion of angiogenic and vasculogenic cytokines such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and fibroblast growth factor-2 (FGF2) [48–50].

The discovery of ASC-administered immunomodulatory effects broadened clinical interest in neurologic and autoimmune disease applications [51–53]. ASCs secrete cytokines and prostaglandins, predominantly interleukin (IL)-4, IL-8, IL-10, and prostaglandin-2, which are implicated in immunosuppression [54,55]. Through expression of these factors as well as selective human leukocyte antigen-II surface expression, ASCs reduce cell-mediated immunity by directing T-regulatory cell expansion [52]. In turn, ASCs have been used in experimental models of graft-versus-host disease, embolic and hemorrhagic stroke, multiple sclerosis and autoimmune encephalitis, spinal cord injury, allergic rhinitis, reactive airway disease, and rheumatoid arthritis [56–65]. Potential results in each of these models have been variable; adipose-derived therapies offer hope and insight into pathologies the cures of which remain obscure to scientists and clinicians alike.

The intravascular administration of stem cells has associated safety and efficacy concerns. Schrepfer et al. demonstrated in mice that within minutes, most BM-MSCs administered intravenously were trapped in the lungs and few cells arrived at the target destination [66,67]. Given the similar size of ASCs, these concerns were quickly transposed to intravenous ASC therapies. Subsequently, the most worrisome adverse event is the inadvertent creation of emboli. Takahashi et al. infused neural stem cells intravenously and did not detect engraftment of cells at the site of injury; worse, up to one-third of the mice died immediately after injection, presumably from pulmonary emboli [68]. Similar results, attributed to the large cell-to-capillary diameter, were obtained using adipose-derived murine ASCs with intraaorta injection [69]. In fact, case reports in humans exist demonstrating pulmonary sequestration and infarction after administration of intravenous adipose tissue-derived cell therapy [70]. There is conflicting evidence, however, regarding the relationship between simple embolism and resultant infarction. In 2011, Ra et al. demonstrated that cell doses as high as 5×10^6 cells/mouse could be administered without an adverse event, so long as the injection was slow enough to avoid bolus injection [71]. These results are similar to those found in BM-MSCs and speak to the absence of ASC-native toxicity. Specifically, with regard to stem cell emboli, Lee et al. found that despite pulmonary sequestration, BM-MSCs increased antiinflammatory protein translation and secretion, specifically tumor necrosis factor-inducible gene 6 [72]. This evidence further bolsters the therapeutic scope of stem cell secretomes and paracrine abilities and provides insight into the mechanism of efficacy without engraftment, safety, and dosing concerns.

The direct, local delivery of ASCs via in situ injection or topical application has been demonstrated successfully as an alternative to systemic delivery, with greater cell engraftment efficiency and mitigation of the necessity for cellular homing as well as first-pass clearance in the lungs, spleen, and liver. In addition, direct application in several clinical models and pathologies has been successful, including myocardial ischemia, peripheral vascular disease, acute and chronic wounds, degenerative joint disease, and inflammatory bowel disease.

Several murine studies have demonstrated not only the engraftment and concomitant expression of requisite cardiac proteins (e.g., myosin, troponin, and transcription factor Nkx2.5) but also improved cardiac function, as measured by stroke volume and ejection fraction, within 1 month after ASC injection into infarcted myocardium [38,73]. Furthermore, the transgenic delivery of hASCs into immunoincompetent mice resulted in engraftment and improved cardiac architecture and function [42]. These findings have also been extended to the SVF and patients with long-standing ischemic cardiomyopathy in the PRECISE trial. Results show that transendocardial injection of syngeneic SVF preparations preserve ventricular function, myocardial perfusion, and exercise capacity [74]. Combined, these results have furthered scientific interest in "cellular cardioplasty" using adipose-derived regenerative cells in the acute and chronic settings.

Many groups have documented the ability of ASC to undergo endothelial regeneration, and when deployed in vivo, they can aid in reestablishing vascular supply and general recovery of limb ischemia [34,46,75,76]. In murine models of hind-limb ischemia, $CD31^-$ ($PECAM\text{-}1^-$) cell inoculation in the proximity of ischemic tissue yielded de novo vessel production. These findings have been repeated and confirmed by independent groups [34,77]. As with other studies, it is postulated that engraftment and as well as its hypoxia-induced secretome (e.g., VEGF, HGF, and FGF2) are complementary in the ASC angiogenic capability [50,78]. Several phase I hASC/SVF trials were undertaken in Europe and the United States. Trial models include chronic limb ischemia in patients with diabetes, with diabetic/venous stasis ulcers, with nonsurgically amenable peripheral vascular disease, and with ASC-coated vascular bypass grafts.

The load-bearing tissue of a joint, the articular cartilage, has limited intrinsic repair capability once damaged, and therefore is a prime target for MSC therapies. Awad et al. in 2002 published data on the in vitro chondrogenic potential of hASCs on various biomaterials including agarose, alginate, and gelatin scaffolds in the presence of chondroinductive growth factors [79]. These studies were confirmed by Lin et al. in 2005 [80]. There has been evidence indicating that ASCs have an inferior chondrogenic potential compared with other MSCs, particularly BM-MSCs [81]. When seeded on a platelet-rich plasma scaffold, ASCs differentiated into functional chondrocytes and secreted cartilaginous matrix, although they were of a reportedly inferior histological quality compared with that of BM-MSCs [82]. However, because ASCs are obtained in large numbers by a minimally invasive procedure, they remain an area of interest for orthopedic pathologies [83]. When used in vivo, ASCs produce significant amounts of cartilage matrix molecules, including collagen type-II and type-IV, and chondroitin 4-sulfate, and subsequently demonstrate on histological, radiological, and biochemical analyses the healing of lapine full-thickness cartilaginous defects when seeded onto a supportive matrix such as fibrin glue [84,85]. Several human trials are being undertaken; a single 2011 case series demonstrated promising radiological and functional outcomes [86]. Intervertebral disks such as cartilage possess a limited regenerative capacity. When cocultured with nucleus pulposus (NP) (the intervertebral disc inner core) cells, ASCs increase extracellular matrix (ECM) production and improve the three-dimensional organization of the NP [87]; in vivo, ASCs have been found to be successful in regenerating experimentally damaged canine and murine intervertebral disks [88,89].

When cultured three-dimensionally and stimulated with osteoinductive growth factors and cytokines, hASCs adopt the characteristics of osteoblasts, including osteocalcin and alkaline phosphatase expression and mineralized ECM [90,91]. In addition, the gene expression profiles can be altered by applying shear stresses, with recapitulation of osteoblastic protein production (e.g., osteopontin and collagen type-$I_{\alpha 1}$) [92]. Collectively, these findings indicate that ASCs can differentiate into mechanosensitive osteoblasts. In vivo use demonstrated that hASCS not only produce osteoid when transplanted into severe combined immunodeficiency mice, they can heal critical-sized calvarial and long-bone defects in dogs, rabbits, mice, and rats [93–96].

Clinical interest in and efforts using ASCs to treat soft tissue wounds and defects continue to accelerate because of the relative unavailability of effective treatments. Some promising evidence of ASC-assisted wound repair has been reported and is likely mediated in large part by its secretome and production enhancement by tissue hypoxia. In 2007, Kim et al. first demonstrated that ASCs accelerate both in vitro and in vivo. When cocultured with human dermal fibroblasts (HDF), hASCs promoted HDF proliferation rates. Moreover, hASC-conditioned media enhanced HDF ECM secretion, specifically collagen type I and III and fibronectin. When these hASCs were mixed with collagen gel solution and injected directly into nude mice wounds, hASCs significantly enhanced wound-edge reepithelialization compared with collagen gel controls [97]. In a follow-up study by the same group, topically

administered ASCs improved reepithelialization rates, an effect of which was contributed to the paracrine ASC secretion of basic FGF, keratinocyte growth factor, transforming growth factor-β, HGF, and VEGF [97]. Other studies confirmed similar wound healing effects of ASCs. One interesting study, completed by Amos et al. in 2009, showed that ASCs applied to wounds in diabetic mice resulted in a faster rate of healing when the ASCs were delivered as multicellular aggregates compared with when the stem cells were delivered as a cell suspension. Furthermore, ASCs formulated as three-dimensional cellular aggregates produced significantly more ECM proteins and secreted higher levels of bioactive factors compared with a monolayer culture [98]. These results emphasize the importance of the cell culture, formulation, and delivery mechanism in the ASC biology and therapeutic effect.

Autologous lipotransfer (also known as fat grafting) was developed over a century ago. In 2008, Yoshimura and colleagues described a process called "cell-assisted lipotransfer," in which ASCs are combined with lipoaspirate in an effort to improve graft viability and long-term volume retention [43]. The presumed mechanisms of reported volume retention compared with controls included progenitor cell differentiation into mature adipocytes and/or improved vascularization and subsequent graft survival. There has been some conflicting evidence in follow-up studies, including a "head-to-head" trial of fat grafting to cell-assisted lipotransfer by Peltoniemi et al. In 18 women undergoing breast augmentation, volume retention trended lower (albeit without statistical significance) in the cell-assisted lipotransfer group as evaluated by magnetic resonance imaging [99]. Further double-blinded, randomized controlled clinical trials are required to establish the superiority of one treatment over another.

Finally, a few groups have used direct application of ASCs in the clinical setting for other therapeutic objectives, including fecal and stress urinary incontinence, inflammatory bowel disease and its fistulous sequelae, erectile dysfunction, Buerger disease, graft-versus-host disease, and spinal cord injury [45]. However, the clinical applications for which ASCs are being used often lack a solid foundation of preclinical data. For example, data demonstrating that ASCs are capable of differentiating outside an adipogenic lineage may require extreme chemical or culture modifications and, on occasion, genetic engineering. Often, "confirmation" of a particular cell phenotype is based on artifactual assays. For example, in ASC-driven osteogenesis studies, alizarin red S assay is frequently used; however, this assay does not distinguish between neo-matrix mineralization and dystrophic calcification caused by dying cells. In addition, if the cells express the characteristic enzyme alkaline phosphatase, it can cleave the donor phosphate group from the medium component, β-glycerophosphate; high concentrations of free phosphate will cause calcium phosphate precipitation, but this mineral in itself is not identical to the natural mineral component of human bony tissue, hydroxyapatite. Nonetheless, the plasticity that ASCs purportedly exhibit, and subsequent scientific interest in them, continue to expand. Continued confirmatory preclinical research efforts are required to validate differentiation potential and an understanding of therapeutic mechanisms of action to ensure that clinical translation is expedient, efficient, and efficacious.

ENGINEERED NEO-TISSUE

ASCs are traditionally cultured on two-dimensional plastic. These culture conditions allow expansion of the putative stem ASC population but do not necessarily mimic the in vivo microcellular environment experienced by a cell: that is, cell−cell and cell−matrix interactions. As focused research on the creation of ex vivo neo-tissue constructs becomes more successful, the ability to recapitulate that microcellular environment becomes increasingly granular, composed of inductive media, growth factors, cytokines, gas concentrations, matrix material and dimension, and even matrix nanodirectional and microdirectional topographies [100,101].

Investigators have used ASCs to repair cartilage, bone, adipose, bladder smooth muscle, trachea, skin, and blood vessels in vitro and in human subjects, using ASCs as the cellular foundation. Starting with a three-dimensional, polylactic-glycolic acid bladder dome combined with an outer porous sponge, Jack et al. seeded the construct with smooth muscle cells differentiated from ASCs and implanted it into rats. After explantation at 12 weeks, histological and functional analyses revealed increasing smooth muscle mass and compliance similar to de novo bladder, respectively [102]. ASCs applied to a collage scaffold and implanted in a rat tracheal defect demonstrated accelerated epithelialization and improved angiogenesis compared with controls and provided an adequate airway [102a]. The ability to tissue engineer skin would be enormously beneficial to patients with thermal injury. Trottier et al. successfully used ASCs in place of dermal fibroblasts to reconstruct the neo-skin's dermal layer. In addition, the authors concluded that using ASCs to create neo-skin resulted in a superior neo-organ because only the samples containing ASCs were found to possess a histologically distinct hypodermis [102b].

The use of a preformed scaffold seeded with cells has become common in tissue engineering paradigms. Because higher cell densities influence cell viability and total matrix production positively, improving neo-tissue survival

requires efficient, homogeneous seeding. This can be challenging, and a number of strategies have been described in the literature. Many groups have published on the seeding of cells onto osteoinductive and/or osteoconductive scaffolds in particular. Janssen et al. described the seeding of human BM stromal cells onto 2- to 6-mm granules of a biphasic calcium phosphate scaffold under both static and perfusion conditions. Static seeding was performed for up to 4 h, but constructs were further cultured for up to 20 days and seeding efficiency was judged qualitatively [103–107]. Silva et al. reported the seeding of bioactive glass scaffolds with cylindrical dimensions of 6 × 3 mm with culture-expanded hASCs using static conditions for 2 h, followed by incubation for up to 21 days, but no specific seeding efficiency was reported [107]. Chen et al. described the seeding of human amniotic MSCs onto porcine gelatin microspheres with diameters ranging from 130 to 380 μm. Their seeding protocol involved cycles of intermittent stirring alternating with static culture for 8 h and resulted in a seeding efficiency of greater than 90% [104]. Frohlich et al. reported the seeding of culture-expanded hASCs onto demineralized bone scaffolds with cylindrical dimensions of 4 × 4 mm. Using repeated pipetting of a highly concentrated cell suspension followed by static culture for 2 days, they report a seeding efficiency of 73% [106]. This was in direct contrast to findings reported by Jurgens et al. with regard to the attachment of SVF cells to scaffolds. They reported that SVF cells attached rapidly to two different scaffold types, but with only 10% efficiency. Interestingly, their findings also suggested that no additional benefit was achieved by prolonging the seeding-incubation step from 30 min to 2 h [106]. It is clear that seeding methods vary widely and efficiency depends on many variables including cell type, scaffold type, and cell concentration, to name a few. These many variables can seem even more daunting when considered in the context of point-of-care objectives. With this approach, constructs are meant to be fabricated in real time at a patient's bedside, in the operating room, or perhaps in a clinic. This reduced time frame adds an additional challenge to an already difficult objective.

Other potential, albeit completely experimental methods of producing engineered neo-tissue constructs using ASCs include techniques involving organ printing and/or explanted microcirculatory beds (EMBs) [108]. Tissue and organ printing may enable large-scale industrial robotic fabrication of living human organ constructs with a "built-in" perfusable intraorgan branched vascular tree (Mironov et al., 2009). These vascularized organ constructs could then be perfused with a solution containing predifferentiated ASCs, which would subsequently engraft and further differentiate, creating a functioning, physiologic neo-organ. However, the ability to regenerate a functional, physiologic, vascularized, three-dimensional organ remains an elusive goal for tissue engineers.

In short, the engineering of neo-tissue constructs from ASCs is at an early stage. The realization of the regenerative potential of ASCs will eventually improve clinical outcomes, but it is dependent upon further advancements in adipose biology including proliferation, differentiation, and signaling.

THERAPEUTIC SAFETY OF ADIPOSE-DERIVED CELLS

Carcinogenesis and Tumorigenesis

Despite a variety of preclinical and clinical studies demonstrating the safety of intravenous and site-directed injection of ASCs, conflicting reports exist indicating the induction or facilitation of tumor growth [109,110]. The resulting concern that multipotent mesenchymal cells, ASCs in particular, could inadvertently effect tumor tropism has surfaced. Proposed mechanisms permitting tumorigenicity/tumor tropism have been postulated to include immunomodulation, inhibition of caspase-induced apoptosis, and increased tumor vasculogenesis. For example, several studies have indicated that ASCs not only home and engraft into tumor and its vascular supply, but augment the tumor growth rate, cellular viability, and metastatic toolbox (i.e., invasion and motility) [110–112].

Because cells respond to the extracellular environment, it is of unsurprising that this milieu is important in the promotion and regulation of cellular functions including the cell cycle, transcription, and translation. It is therefore entirely possible that ASC-driven tumor tropism is caused in large part by its secretome and its paracrine–endocrine effects. Studies isolating the effects of this secretome using ASC-conditioned media (but devoid of adipose-derived cells) have demonstrated the ability of this conditioned media to increase cytokine and growth factor production of endometrial and breast cancer cells while simultaneously increasing tumor proliferation rates [113,114].

Other research directly contradicts the tumor-promoting behavior of ASCs. The proliferation of primary leukemia cells is inhibited by secreted Dickkopf-related protein 1 (DKK-1), the transcription of which is actively regulated via the ASC transcription factor NANOG [115]. In addition, ASC coculture studies of ASC and breast cancer cells report statistically significant reductions in cancer proliferation and metastatic rates, reportedly via the same DKK-1

expression [116]. Other in vivo modeling recapitulates these outcomes but is often attributed to other mechanisms, including downregulation of cell-cycle progression [117].

There is a large and growing number of humans who have received MSC therapies (both BM- and adipose-derived cells as well as allogeneic and autologous cells) for a variety of clinical indications, by a variety of delivery routes: for example, the RESTORE-2 trial, a prospective clinical trial using autologous ASC-enriched fat grafts to reconstruct postmastectomy contour defects. With only 2 of 71 total patients lost to follow-up, there was no reported local cancer recurrences at the 12-month end point [118]. Furthermore, the current authors are unaware of any reported oncologic-related adverse events associated with any human adipose cell-derived studies or therapies. Nevertheless, there is no doubt that the (putative) oncologic risks associated with regenerative cell therapies deserve focused and continued thorough evaluation. Moreover, the mantra of "First do no harm" must guide a clinician's activities no matter how noble the intent. On the other hand, worthy efforts to advance and translate the field of regenerative medicine will inevitably be associated with risks inherent to the new and unknown, even in light of thorough and meticulous science, and the extensive education and comprehensive and ethical consent of courageous, sometimes desperate patients.

CONCLUSIONS

Attempts to harness the pluripotent and paracrine potential of lipoaspirate cells (i.e., the heterogenous SVF and culture-expanded ASC) are increasing in number, and clinical translation of adipose science is in a fledgling but quickly growing stage. The mechanisms through which adipose-derived cells modify the extracellular micromilieu (e.g., secretome paracrine signaling, cell−cell interaction and induction, cellular homing, and engraftment) continue to be studied and elucidated, and with this expansion of solid, preclinical knowledge, further clinical discovery and subsequent therapeutic promise will continue to be realized. The relative accessibility of autologous adipose and abundance of stromal cells and putative progenitors facilitate these studies and subsequent scientific progression. However, current regulatory rules are not globalized, and they often depend on country-specific guidelines. As the understanding of adipose stromal cell biology becomes transparent, the maturation of policies governing their human clinical applications will soon follow. ASCs will likely become a cellular keystone in the field of regenerative medicine and improve our ability to treat countless diseases and disorders.

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CHAPTER

20

Peripheral Blood Stem Cells

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INTRODUCTION

Adult stem and progenitor cells have been isolated from a wide variety of sources. Lineage-committed progenitors are found in a number of tissues including skin, fat, muscle, heart, brain, liver, pancreas, and bladder. Adults have another source of stem and progenitor cells is are not restricted to a specific tissue. These "universal" stem and progenitor cells are found circulating in peripheral blood (PB), which allows them to reach and integrate into all tissues.

The various hematopoietic stem cells (HSC), and nonhematopoietic stem cells, and progenitor cells found within the PB are thought to originate primarily from the bone marrow (BM) in adults. Hemangioblasts are the embryonic precursors of HSC, which develop into the progenitors and mature cells of all of the various hematopoietic lineages. These progenitor cells complete their differentiation in the BM, PB, and thymus. Extensive research in hematology and oncology has resulted in the identification of a wide variety of cell surface markers that allow the characterization and isolation of hematopoietic cells at different stages of differentiation and lineage commitment. Initially, adult BM-derived mesenchymal stem or marrow stromal cells (MSC) were isolated, expanded in vitro, and examined for their multilineage differentiation potential. These early studies were followed by extensive research showing that this unique cell population can proliferate extensively in vitro without loss of potential, differentiate to multiple lineages in vitro and in vivo, and contribute to the regeneration of several tissues in vivo [1]. Like HSC, it is possible that MSC may leave the BM environment and circulate within the PB. Identification and isolation of MSC is based on the differential expression of cell surface markers that distinguish them from circulating HSC. Endothelial progenitor cells (EPC), another progenitor population within the PB, are derived from the same hemangioblast precursors as HSC, but they take a separate path of differentiation in the BM. The identification of circulating EPC suggested that the process of vasculogenesis, which was previously believed to be restricted to the embryonic stages, continues into adulthood. Circulating EPC have specific cell surface markers that are unique to their primitive state and are lost when EPC differentiate into mature endothelial cells (EC).

This chapter will briefly review the types and source of stem cells in PB, their specific cell surface markers, and factors that change their abundance in PB. We will focus on the isolation and in vitro expansion of MSC and EPC and describe their therapeutic applications for regenerative medicine. We will further describe the role of PB-derived stem cells in normal and pathological processes. Although much information has been gathered on the identification of different populations of PB stem cells, their clinical potential for therapy is just now being explored. Because PB is readily obtainable, their consideration as a viable source of cells for regenerative medicine deserves special attention.

TYPES AND SOURCE OF STEM CELLS IN THE PERIPHERAL BLOOD

All of the countless cells of all 10 hematopoietic lineages (erythrocytes, platelets, neutrophils, eosinophils, basophils, monocytes, T and B lymphocytes, natural killer cells, and dendritic cells) that are found within the circulation at any given time are produced within BM through the carefully orchestrated process known as

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hematopoiesis. In the adult, the process of hematopoiesis is supported by a rare population of cells within the BM known as HSC. Although HSC account for only about 0.01% of the total BM cellularity [2], it has been estimated that under steady-state conditions, this remarkable process must generate a remarkable 10^{10} erythrocytes and 10^{9} leukocytes each hour in a human while maintaining the circulating levels of each cell lineage within tightly regulated limits. Moreover, the BM can respond on-demand rapidly to increase the production (and release into the circulation) of the specific lineage(s) needed to counter a variety of insults and injuries, ensuring restoration of homeostasis. Although in the absence of disease BM remains the primary site of hematopoiesis throughout life [3], as early as the 1960s, it was recognized that small numbers of HSC are present at all times, circulating within in the PB stream as they transit from one site in the marrow to another, thus ensuring that the marrow is continuously and homogeneously "seeded" with these cells [3]. Interestingly, the levels of circulating HSC have been shown to fluctuate throughout the day, paralleling changes in Circadian rhythm, and increase in response to exercise and a variety of environmental and chemical stressors [4]. This process, known as “mobilization,” will be discussed in detail in the next section, covering both its mechanism(s) and clinical implications.

Although most of the cells in the PB are hematopoietic in origin, other nonhematopoietic stem/progenitor cells are present. The most abundant of these are EPC, which derive from the same precursor during ontogeny as the HSC: the hemangioblast. Throughout life, EPC participate in repairing endothelial damage arising from injury and disease, and can also contribute to vasculogenesis, a process that was previously thought to be restricted to embryogenesis. As a result, many preclinical and clinical studies are exploring the use of EPC to treat ischemia in various tissues arising as a result of a range of injuries and diseases. Like HSC, the levels of EPC in the PB can fluctuate fairly widely in response to a variety of factors and stressors, likely also as a result of the mobilization of reserves of EPC from the BM. These findings have raised the hope that it may be possible to trigger or enhance the release of endogenous EPC for therapeutic purposes.

Another important BM-derived stem/progenitor cell is the MSC. MSC reside in the perivascular regions of the hematopoietic niche of the BM, from which they provide a wide array of physical and chemical signals that are required to drive or regulate the hematopoietic process. MSC or MSC-like cells also exist throughout the rest of the body as pericytes in close association with the vasculature. MSC released from the BM or these pericyte reserves have been reported to have a key part in maintaining and repairing damaged tissues throughout life, and there have been a handful of reports describing the presence of MSC in the PB [5]. However, their presence in significant numbers and their ability to be “mobilized” are still contentious. Nevertheless, given their emerging role as cellular therapeutics in regenerative medicine and as vehicles for gene delivery, we will discuss these cells in detail in this chapter.

Mobilization of Bone Marrow Cells

Hematopoietic Stem/Progenitor Cell Mobilization

When HSC transplantation was first adopted as a clinical procedure, limited knowledge of the hematopoietic system at the time dictated that BM serve as the source of the HSC graft. To collect HSC for these early transplants, a substantial volume of BM had to be harvested from the pelvis or sternum under general anesthesia. While investigators were fine-tuning the details of conditioning regimens and graft composition, compelling evidence emerged that the absolute number of HSC present within the BM graft that was transplanted strongly correlated with the robustness of hematopoietic engraftment. Moreover, increasing the dose of HSC resulted in significantly lower mortality from infectious complications posttransplant [6]. These important realizations fueled the search for methods to increase the number of HSC that could be harvested for transplant.

Before it was possible to begin devising newer and more efficient ways to increase the numbers of HSC that could be obtained in a graft, a better understanding was necessary of the interactions between HSC and the BM niches in which they normally reside. Studies over the past decades revealed that the most primitive HSC are maintained in the BM niches by binding to the extracellular matrix (ECM) and stromal cells of the BM via multiple adhesive interactions [7]. Of these, binding of the ligands stromal-derived growth factor-1 (SDF-1α) (also known as CXCL12) and vascular adhesion molecule-1 (also known as CD106), which are expressed on the surface of cells in the BM microenvironment, to their respective receptors, CXCR4 and very late antigen-4 (also known as $\alpha 4\beta 1$-integrin), which are expressed on the surface of HSC, have emerged as essential signals for the retention of HSC in the BM niches and in the trafficking of HSC to and from the BM [8,9]. As early as the 1960s, it was recognized that HSC residing in BM could be released from their niches and circulate in the PB, albeit at low levels, under steady-state homeostasis [10]. In subsequent years, it was observed that the levels of these circulating HSC often increased after chemotherapy.

Further studies demonstrated that the levels of naturally circulating HSC follow changes in circadian rhythm [11,12] and that the number of circulating HSC increases in response to a wide array of physiological stimuli and

stressors, including systemic or local inflammation, strenuous exercise, psychological stress, and tissue or organ injury [8,9]. This process of so-called "mobilization," in which HSC are coaxed to leave their niches within the BM and enter the circulation, is still not well-understood but has been postulated to be orchestrated by (1) SDF-1α degradation and inactivation within the BM niches by proteolytic enzymes such as elastase, cathepsin G, and various metalloproteinases (MMP); (2) neurotransmitters released from nerves that innervate the BM microenvironment; and (3) reversal of the transendothelial chemotactic gradient between the BM microenvironment and plasma [13]. Evidence has also accumulated that the exodus of HSC from stem cell niches is significantly augmented in a complement cascade (ComC)-dependent manner [9]. The ComC is activated by the classical, mannan-binding lectin and alternative pathways. Activation of the ComC and generation of cleavage fragments of the fifth component of the ComC (C5) initiate events that are required for egress of HSC from BM into PB [14]. Further studies have provided compelling data to support the conclusion that the coagulation cascade is activated in parallel with the ComC during mobilization and that it has a supporting role in this process [13]. Additional support for the importance of the ComC in mobilization is provided by the fact that the ComC is activated in all of the mechanisms leading to mobilization of HSC (for example, systemic inflammation, infection, organ injury, stress, and strenuous exercise) [9].

In addition to these physiological triggers, studies in the 1970s and 1980s revealed that administering pyran copolymer to mice or dogs could induce primitive colony-forming HSC to "mobilize" from the BM into the circulation [15,16]. Moreover, the dose and number of times or frequency with which pyran copolymer was administered directly affected the resultant levels of circulating HSC. These findings led the authors to conclude that mobilized PB (mPB) could represent a viable source of HSC for transplantation. Subsequent studies showed that hematopoietic cytokines could mobilize primitive HSC with long-term repopulating ability into the PB with relatively high efficiency [17], and in the early 1980s, the first autologous transplants using HSC from mPB were reported [18]. After the discovery and clinical development of human granulocyte colony-stimulating factor (G-CSF) and the realization that immunoisolation could be used to obtain highly enriched primitive HSC from this cytokine-mPB, thereby removing malignant cells and making it possible to use autologous cells to treat hematological malignancies [19], G-CSF quickly become the standard and most widely used mobilizing agent for autologous HSC transplantation. It has been the standard of care for the past several decades. G-CSF is thought to mobilize HSC from the BM niche primarily by stimulating the secretion of neutrophil-associated extracellular proteases, such as matrix MMP-9, which can degrade SDF-1α, thereby releasing HSC from their niche [20]. Experiments with neutralizing antibodies directed against CXCR4 or SDF-1α have provided further evidence that uncoupling of the SDF-1α—CXCR4 axis is critical for G—CSF—mediated HSC mobilization.

The noninvasiveness and ease with which mPB could be collected (compared with the general anesthesia required for BM harvest and the pain that follows multiple aspirations from the BM), and the discovery that HSC were present at higher frequency in these mobilized products than in steady-state BM, quickly led multiple laboratories and centers around the world to begin replacing BM with mPB in the clinical setting [21,22]. Indeed, the use of mPB as an HSC source has dramatically increased and now accounts for approximately 75% of HSC transplants from unrelated adult donors [6] and about 99% of autologous HSC transplants [23]. Interestingly, this dramatic shift has taken place without much hard clinical data to support the superiority of mPB in terms of better patient outcome or safety [6], and despite concerns that the higher T-cell content present in mPB might lead to higher risk for graft versus host disease (GVHD) [24]. Support for this shift has come from numerous large, multicenter, randomized trials that have collectively shown that engraftment or reconstitution is often faster and more robust with mPB than with BM, shortening the duration of cytopenias, and that the use of mPB may be associated with a decreased relapse rate, but this appears to depend on the patient population and malignancy in question [25]. In the allogeneic transplant setting, the use of HSC from mPB, compared with those from BM, was shown to be associated with more rapid engraftment, reduction of infectious complications, and lower transplant-related mortality in patients with advanced hematologic malignancies [21]. However, as originally feared, these studies also revealed that the use of mPB is associated with a significantly higher rate of chronic (and perhaps acute) GVHD than BM and offers no significant improvement in survival [26].

Despite the marked clinical success of G-CSF and the dramatic change it has enabled in the practice of HSC transplantation, 5%—20% of individuals fail to mobilize a sufficient number of HSC into the PB in response to G-CSF, for reasons that are unclear. This inability to mobilize a significant percentage of patients requiring HSC transplantation has led investigators and clinicians to seek alternative means of mobilizing HSC. Besides G-CSF, other cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-7, interleukin-3, interleukin-12, stem cell factor, FLT-3 ligand, and interleukin-8 have been reported to mobilize HSC clinically or experimentally in animal models [27]. However, each of these molecules differs in the time to achieve efficient mobilization, the type of cells it mobilizes into the PB, and its side effects.

Plerixafor/AMD3100 is a bicyclam molecule that specifically and reversibly blocks SDF-1α binding to CXCR4 [28,29]. It was initially developed as an antagonist of the CXCR4 HIV coreceptor that is present on the surface of $CD4^+$ T cells, thereby blocking HIV cell entry. Subsequently, however, this agent was demonstrated to possess significant HSC-mobilizing activity. This is unsurprising because inhibition of this critical HSC–niche interaction induces the rapid egress of HSC from the BM into the peripheral circulation. Indeed, whereas cytokines induce HSC mobilization with delayed kinetics (over the course of days), mobilizing with agents such as Plerixafor that directly disrupt the critical SDF-1α–CXCR4 axis can generate peak levels of mobilized HSC within only hours of administration.

In addition to its much faster action, Plerixafor has also been shown to mobilize a larger absolute number of $CD34^+$ cells in patients who have failed to yield an adequate amount of $CD34^+$ cells upon mobilization attempts with G-CSF with or without chemotherapy. Importantly, compared with G–CSF–mobilized cells, a higher percentage of Plerixafor-mobilized $CD34^+$ cells are in the G1 phase of the cell cycle and belong to the more primitive $CD34^+/CD38^-$ subset of cells. Limiting dilution HSC transplant studies in animal models showed that Plerixafor-mobilized HSC have a higher frequency of true HSC with long-term repopulating activity [28]. Thus, in addition to an absolute increase in overall $CD34^+$ cells upon Plerixafor mobilization, a more primitive and potent progenitor cell population is also significantly elevated. Numerous clinical trials have demonstrated that combining G-CSF and Plerixafor can further increase the $CD34^+$ cell yield; they also showed that a higher proportion of the mobilized progenitors possesses the primitive and possibly more potent $CD34^+/CD38^-$ phenotype.

Studies over the past several years, largely spearheaded by the Ratajczak group, have provided greater insight into the mechanisms by which HSC are mobilized and have begun to provide possible explanations for why certain people are so-called "poor mobilizers." Studies by this group showed that heme oxygenase-1 (HO-1), which has well-documented antiinflammatory potential, has an important and previously unrecognized role in the retention of HSC in BM niches. HO-1 appears to exert this effect through several different means, the most important of which are: (1) negatively modulating activation of ComC; (2) maintaining SDF-1α levels in the BM microenvironment; and (3) attenuating the chemotactic responsiveness of HSC to SDF-1α and sphingosine-1 phosphate gradients in the PB. These investigators also showed that HO-1 activity inhibits HSC mobilization in response to G-CSF or Plerixafor/AMD3100 [30]. Further study revealed that upregulation of inducible nitric oxide (NO) synthase enhances HO-1 activity, thereby acting as a novel negative regulator of hematopoietic cell migration and preventing egress of HSC into PB during mobilization [31]. This group also made the astute and important observation that all of the processes that have been shown to induce HSC mobilization also trigger activation of several ancient proteolytic cascades, such as the ComC [9,14], the coagulation cascade, and the fibrinolytic cascade [32]. The researchers found that when HO-1 metabolizes heme, it produces molecules with potent antiinflammatory properties, including inhibition of the ComC. Intriguingly, mutations in the ComC pathway occur at a frequency similar to the observed rate of "poor responders," which strongly suggests that this pathway is critically involved in this important clinical problem.

Mobilization of Other Bone Marrow–Derived Cells into Peripheral Blood

Studies have provided compelling evidence that many of the same physiological stressors such as injury and intense exercise and pharmacological agents such as G-CSF and Plerixafor/AMD3100, which mobilize HSC efficiently from the BM niches into the circulation also mobilize EPC, and that this mobilization can create pronounced therapeutic benefit in a range of diseases [33]. However, although many of the pathways appear to be shared between HSC and EPC, such as the involvement of MMP-9 [34] and regulation by NO [35], the optimal parameters or combination of cytokines or growth factors to induce mobilization differ between HSC and EPC [36]. In addition, EPC are also mobilized in response to stress signals such as hypoxia-inducible factor-1α [37] and hormonal changes that occur during the menstrual cycle [38], which do not mobilize HSC.

In addition to EPC, another important BM-derived stem/progenitor cell is the MSC, the biology and properties of which will be discussed in detail in a subsequent section. Current thought holds that MSC released from the BM or other perivascular reserves throughout the body have an important part in maintaining and repairing damaged tissues throughout life. To accomplish this task, one would assume that they must transit to areas of damage or disease via the bloodstream. However, the detection of MSC in the PB has proven difficult, and there have only been a handful of reports actually describing the presence of MSC in the PB and their mobilization in response to injury or pharmacological agents [5]. As a result, their presence in significant numbers and their ability actually to be mobilized are still contentious. Indeed, other studies have shown a lack of mobilization, but rather alterations to the MSC secretome in response to stressors that provoke HSC and EPC mobilization [39].

ENDOTHELIAL PROGENITOR CELLS

Initial evidence that EPC can be detected in PB came from research conducted mainly by the groups of Isner and Asahara in Boston and Rafii in New York [40,41]. They showed that cells with EC characteristics can be isolated from PB and expanded in vitro. They and others showed that the numbers of EPC in PB were significantly increased as a result of acute vascular injuries, angiogenic stimuli, estrogen, and NO synthase but were reduced by certain chronic disease states (e.g., coronary artery disease) [42]. Circulating EPC originate primarily from the BM and can be identified by the differential expression of hematopoietic and EC markers. This is important because hematopoietic and EPC probably share a common precursor, the hemangioblasts [43]. Hemangioblasts reside mainly in the BM and differentiate into HSC and angioblasts. This process occurs mainly during early embryogenesis but was shown to exist in adults [42,44]. Angioblasts will develop into EPC that, upon stimulation with angiogenic factors such as vascular endothelial growth factor (VEGF) and placental growth factor, are mobilized from BM to PB [42,45]. Once in PB, EPC can be recruited to sites of active neovascularization such as those seen in wounds, diabetic retinopathy, and tumors [46]. The role of EPC in physiological and pathological neovascularization and their therapeutic applications are described next.

Identification and Isolation of Endothelial Progenitor Cells

Marrow and PB cells expressing CD34 can develop into EPC [40,47]. Although CD34 is commonly used to isolate EPC, CD34 expression is also shared by HSC and MSC and cannot be used to distinguish between these populations. Likewise, VEGF receptor 2 (VEGFR-2) (human kinase insert domain receptor [KDR] and mouse Flk-1), which is used to identify EPC, is expressed also on HSC [40]. In humans, CD133 (AC133) is used to distinguish EPC from mature EC, because CD133 is not expressed by mature EC [47]. CD133 is a stem cell marker with functions that have not yet been recognized [48]. In addition, Hebbel and colleagues have used P1H12 antibodies that recognize CD146 (MUC18) on circulating EC in PB but not on monocytes, granulocytes, platelets, megakaryocytes, or T or B lymphocytes [49]. Other markers common to progenitor and mature EC are the cell surface receptors KDR and Tie2 [46]. Purified populations of $CD133^+/KDR^+$ EPC proliferate in vitro in an anchorage-independent manner and can be induced to differentiate into mature adherent EC [46]. It is thought that $CD133^+/KDR^+$ EPC are a population of immature EC that are mobilized from the BM to participate in neovascularization. Because myelomonocytic cells have lost surface expression of CD133, this marker also provides an effective means to distinguish true EPC from cells of myelomonocytic origin. Yet, studies showed that cells expressing CD14, considered to be a typical monocytic lineage marker, can become EC [50]. Collectively, these studies suggest that identification of circulating EPC may be achieved using different markers that may define subpopulations of EPC based on their stage of differentiation and origin.

The number of EPC in BM is low (<10 per 10×10^5 mononuclear cells [MNC]) and the reported numbers vary based on which identifying markers are used among the different studies. For practical applications, the EPC fraction may be enriched using cell surface markers such as CD34, CD133, and KDR [40]. SDF-1 stimulates the mobilization of EPC via an enhancement of protein kinase B (Akt) and endothelial NO synthase (eNOS) activity [51]. Interestingly, VEGF has been found to promote EC to express SDF-1 and CXCR4 (the SDF-1 receptor) [52]; on the other hand, SDF-1 has the potency to induce the expression of VEGF [53]. Thus, in the BM vascular niche, the expression of VEGF and endothelial SDF-1 possibly modulate the microenvironment through proteolytic enzymes (MMP-2 and MMP-9) and by controlling EPC mobilization. In addition to these factors, data indicate that erythropoietin (Epo) [54], platelet-derived growth factor (PDGF) [55], and NO [54] stimulate EPC mobilization.

One functional assay capitalizes on in vitro growth kinetics to discriminate BM-derived EPC and circulating EC from vessel wall–derived mature EC [46]. In this assay, the isolated cells are incubated with VEGF, basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), and fibronectin or collagen. EC colonies that appear early are derived from the recipient vessel wall circulating EC, whereas late-outgrowth cells or colonies originate mainly from BM-derived EPC. Lin et al. [56]. and Gulati et al. [57]. reported that early EPC (7-day cultures) are derived from $CD14^+$ cells (monocytes), whereas late-outgrowing EPC (4–6 weeks of culture) are derived from $CD14^-$ cells that bear endothelial markers ($VE\text{-}cadherin^+Flk\text{-}1^+vWF^+CD36^+CD146^+$). In addition, Hur et al. [58] cultured total MNC obtained from human PB and reported two EPC types. Some cells organized into clusters as early as 3 days after plating; these were called early EPCs. These cells had limited proliferative capacity and disappeared after 4 weeks of culture. In contrast, another group of cells appeared later, at 2–3 weeks after plating. These cells demonstrated EC-like cobblestone morphology and were called late EPC. Late EPC proliferated robustly and

were positive for VE-cadherin, Flt-1, and KDR but were negative for CD45. The late EPC produced NO and formed capillary tubes. Yoder's group [59] reported the late outgrowth of cells from umbilical cord or circulating blood MNC that they termed endothelial colony-forming cells (ECFC). These cells appeared at 14–21 days after plating and formed adherent colonies with cobblestone morphology. ECFC expressed the cell surface antigens CD31, CD105, CD144, CD146, vWF, and KDR and took up acetylated low-density lipoprotein (AcLDL). ECFC did not express hematopoietic or monocyte/macrophage cell surface antigens such as CD14, CD45, or CD115. Whether isolated from cord or adult PB, ECFC display clonal proliferative potential and relatively high levels of telomerase [60]. Taken together, the consensus is that late-outgrowth endothelial colonies may be considered to be angioblast-like EPC.

The cell surface antigen CD31 (also known as platelet endothelial cell adhesion molecule-1) has been used as a marker for EC for many years [61]. It was suggested that CD31 could also be used as a marker for EPC [62]. However, because it has been shown that $CD31^+$ are fully differentiated EC, it could be argued that CD31 may not be a suitable marker for identification of EPC, because it may not distinguish between progenitor and fully differentiated EC. However, in those studies, CD31-selected EPC had a typical EC/EPC phenotype, proliferated robustly, and integrated into nascent vasculature. The close phenotypic similarity between mature and progenitor EC was highlighted by Ingram et al. [60]. Those researchers used single-cell colony-forming assays to demonstrate that populations of both human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAEC), which were thought to be fully differentiated EC, contain a subpopulation of EPC with different clonogenic and proliferative potential. That work suggested that HUVEC and HAEC are not homogeneous populations and appear to contain some fully differentiated EC that are unable to proliferate, as well as other cell fractions that demonstrate more "stemness," as shown by their robust ability to proliferate and form colonies. It is not yet clear why HUVEC and HAEC populations demonstrate heterogeneity in ex vivo culture conditions, but these findings suggest that culturing and sorting for EPC may standardize experimental data obtained from different research groups.

Isolation of EPC is conventionally based on fluorescence-activated cell sorting or immunomagnetic techniques, which are complex, time-consuming procedures that need an experienced operator. New devices could be engineered to simplify the operation and shorten the time for obtaining EPC. Miniaturization of EPC isolation procedures by using similar strategies, adopted from microfluidic lab-on-a-chip devices, may significantly facilitate EPC isolation [63]. Plouffe et al. [64] developed a microfluidics chamber, coated with antibodies against CD34, VEGFR-2, CD31, CD146, or CD45, to capture EPC. When cells were flowing through the "EPC chip," they demonstrated specific affinity to EPC. Although this prototype device can be coated with only one antibody on a single chamber, it is potentially an alternative for easy and rapid separation of EPC. Tillman et al. [65] developed an extracorporeal cellular affinity column that can recover CD133-expressing progenitor cells with high efficiency. In a sheep model, 1.8 L of blood was passed through a Sepharose-based column with affinity for CD133 and unbound cells and plasma were returned to the animal. The results showed that this process had a minimal effect on the hematologic and physiologic parameters of the animal and EPC recovery was over 600-fold more efficient than conventional density centrifugation from a PB specimen. This technology may facilitate the generation of large numbers of progenitor-derived cells for clinical therapies and reduce the time required to attain clinically relevant cell numbers while minimizing loss of other important cell types to the donor.

In Vitro Expansion of Endothelial Progenitor Cells

The relative paucity of EPC in the circulation likely contributes to the difficulty associated with EPC culture [60]. Density-gradient separation procedures, designed for separating MNC from blood, may contribute to this problem. Thus, successful culture of EPC usually requires a relatively large amount (50–100 mL) of peripheral or cord blood, and yet few colony-forming units (CFU) or ECFC can be obtained (see Table 20.1 in [59]). The mononuclear fraction is placed in fibronectin-coated plates containing endothelial basal medium with contain angiogenic growth factors such as VEGF and bFGF. Other growth factors such as epidermal growth factor and IGF contribute to cell growth but not differentiation. In one of their earlier studies, Asahara et al. [66] showed that VEGF and not bFGF is important for EPC differentiation, and bFGF may be used by the differentiated EC for subsequent proliferation. Inclusion of angiogenic factors in the media prevents "contamination" by other cell types including lymphocytes, macrophages, and dendritic cells. VEGF appears to inhibit dendritic cell maturation from $CD34^+$ MNC fraction [67]. Within 7–10 days of culture in fibronectin- or collagen-coated dishes, colonies with spindle-shaped cells appear in the dish. These are "slow-growing" cells, defined as "late-outgrowth" EPC. They differ from the mature

TABLE 20.1 Cell Surface Markers Expressed on Progenitor and Mature Endothelial Cells

	Putative Endothelial Progenitor Cells	Vessel Wall–Derived Circulating Endothelial Cells
Proliferative capacity:	**High**	**Limited**
Proposed source and mechanism for mobilization:	**Bone marrow; release and proliferation to VEGF and other stimuli**	**Endothelial cell damage; VEGF decreases apoptosis**
MARKERS		
VEGF receptor-2 (kinase insert domain receptor)	+	+
CD34	+	+
CD31 (platelet endothelial cell adhesion molecule)	±	+
AC133 (CD133)	+	−
MUC18 (P1H12)	−	+

VEGF, vascular endothelial growth factor.

circulating EC that readily proliferate in vitro [42]. The whole-blood culture method may be used to increase the likelihood of obtaining EPC cultures. This approach does not require MNC separation procedures, which may minimize cell damage caused by density gradient separation. Reinisch et al. [68] reported that whole-blood culture yielded nearly eightfold more ECFC colonies compared with density gradient separation. The whole-blood culture approach may simplify EPC culture procedures and increase the possibility that EPC will be used for clinical diagnostic and therapeutic purposes.

Commercially available media kits were introduced to facilitate EPC growth. These kits provide reagents and media for culturing EPC from PB. However, these culture kits may not be optimal for EPC expansion and differentiation. Hur et al. [58]. reported that cells isolated with such kits displayed limited proliferative capacity and did not integrate into nascent vasculature [69]. Further studies revealed that monocytes and T cells can contribute to CFU EC [70]; therefore, it is reasonable to argue that some of these kits are not suitable for EPC culture and assays. Instead, ECFC [59] should be cultured in endothelial cell growth medium-2 culture medium, which was developed from MCDB131 medium in the 1980s [71]. ECFC demonstrate late colony-forming, robust proliferation, endothelial morphology, the expression of EPC/EC-related markers, and the ability to integrate into nascent vasculature. Regardless of the EPC source, the cells assume a typical flat EC morphology after 2–3 weeks and present mature EC markers such as CD31, VE-cadherin, and CD146 (P1H12). They metabolize AcLDL, bind *Ulex europaeus* agglutinin-1, and produce NO, consistent with EC properties. Proper characterization of EPC-derived EC requires the analysis of a combination of cell surface markers.

The Role of Endothelial Progenitor Cells in Physiological and Pathological Neovascularization

Blood vessels form by two processes: (1) angiogenesis, or the sprouting of capillaries from preexisting blood vessels; and (2) vasculogenesis, or the in situ assembly of capillaries from undifferentiated EC. Vasculogenesis takes place mostly during the early stages of embryogenesis [72]. Vascular channels in the yolk sac originate from the mesoderm by the differentiation of angioblasts, which subsequently generate primitive blood vessels [73]. Early findings that EPC can participate in angiogenic processes indicate that postnatal neovascularization does not rely solely on sprouting from preexisting blood vessels (angiogenesis) but may be assisted by EPC via postnatal vasculogenesis [66].

BM-derived EPC contribute to adult tissue neovascularization in several models including wound healing, cornea, and tumor angiogenesis [66]. BM-derived EPC may be detected in normal organs including spleen, lung, liver, intestine, skin, hind-limb muscle, ovary, and uterus, which indicates their participation in maintaining

physiological neovascularization [66]. Hormonally induced ovulation cycles were also associated with the localization of BM-derived EPC in corpus lutea and in the uterus endometrium and stroma. These findings indicate that EPC contribute to physiological neovascularization associated with the postnatal regenerative processes.

Minami et al. [74] examined the presence of endothelial, smooth muscle, and Schwann cell chimerism in patients with sex-mismatched (female-to-male) heart transplants. The Y chromosome was used to determine chimerism. Biopsy specimens taken at subsequent times after heart transplantation showed that EC had the highest degree of chimerism (24.3%), Schwann cells showed the next highest chimerism (11.2%), and vascular smooth muscle cells (SMC) had the lowest (3.4%). Results of this study indicated that circulating progenitor cells are capable of repopulating most major cell types in the heart, but they do so with varying frequency. The signals for endothelial progenitor recruitment occur early and could relate to the injury during the surgery.

Cumulative evidences show that BM cells may improve ischemic myocardium function by paracrine stimulation of angiogenesis rather than differentiation into contractile cardiomyocytes [75]. In addition to contributing to neovascularization directly and indirectly, EPC may also provide paracrine survival signals to cardiomyocytes [76]. Clinical studies also suggested that cell-based therapy with EPC can improve myocardial function [77]. In parallel, EPC were found to be incorporated into the vasculature of pathological lesions such as atherosclerotic plaques, tumors, the retina, and ischemic brain tissue. Vascular SMC proliferation results in neointimal hyperplasia and the development of restenosis. BM-derived SMC can integrate into the hyperplastic neointima and atherosclerotic plaques [78]. Evidence for the contribution of BM MSC to human atherosclerotic plaques originated from a study showing donor-derived neointimal cells within the plaques [79]. Also, decreased EPC in the circulation have been correlated with a higher risk of cardiovascular complications [80]. It was hypothesized that lower levels of PB-EPC were associated with an impaired capacity to repair the damaged vessels, but the pathophysiological role of BM-derived EPC remains unclear. Recruitment of PB-EPC to damaged or diseased tissues depends on the underlying pathology and probably results from the release of specific growth factors and chemokines by these tissues [81]. Abnormal retinal neovascularization contributes to the pathogenesis of proliferative retinopathy in diabetes and age-related prematurity and macular degeneration. BM-derived hemangioblasts were shown to contribute to retinal neovascularization in models of proliferative retinopathy [82,83]. This study documented the incorporation of EPC into mature endothelium of the retinal blood vessels. Cerebral infarction is associated with neovascularization of the ischemic zone and new vessel growth. BM transplantation studies showed that EPC could be detected in the neovessels at the repair sites after 3 days [84]. EPC reduction is also considered to be a mechanism by which cardiovascular risk factors promote cardiovascular disease (CVD). A decrease in EPCs is also associated with the pathophysiology of rheumatologic disorders [85]. In this context, restoration of EPC function may prevent long-term cardiovascular complications associated with cardiovascular risk factors and attenuate clinical manifestations of rheumatic diseases.

Whereas restoring EPC function in CVD may prevent disease development, targeting EPCs in cancer may have potential therapeutic benefits. EPC recruitment during tumor progression contributes significantly to the cancer-associated neovasculature. Thus, targeting these cells could be used effectively to halt tumor progression by preventing vascularization of neoplastic deposits [85]. Studies have shown a correlation between increased VEGF and mobilization of EPCs to tumor sites [86,87], whereas inhibiting VEGF receptors halts tumor growth in vivo [88]. Other factors that have an integral role in mobilizing EPCs to tumor sites include interleukin-8 and NO [87].

Compelling evidence for the role of EPC in tumor vascularization comes from a study by Lyden and colleagues using an angiogenesis-defective mouse model. Mice lacking both alleles of Id1 ($id1^{-/-}$) and Id3 ($id3^{-/-}$) died by embryonic day 13.5 and exhibited massive vascular malformation [89]. The $Id3^{-/-}/id1^{+/-}$ mice survived but could not support the growth of several tumor types owing to insufficient tumor vascularization. However, transplantation of $id3^{-/-}/id1^{+/-}$ mutant mice with BM from wild-type mice resulted in tumors that were indistinguishable from tumors grown on wild-type mice [90]. Furthermore, 90% of the tumor vessels contained BM-derived EC, which indicated the contribution of EPC to tumor neovascularization. VEGF treatment failed to elevate the number of EPC in $id3^{-/-}/id1^{+/-}$ mutant mice but not in $id3^{-/-}/id1^{+/-}$ transplanted with wild-type BM. Further evidence was provided by a model in which transplantation of human BM-derived multipotent adult progenitor cells into tumor xenograft-bearing mice resulted in the incorporation of human cells as 40% of the tumor vessel endothelium, which indicated the importance of circulating endothelial cells (CEC) for tumor neovascularization [91]. Different tumors secrete different types and concentrations of angiogenic factors that may have a different capability of inducing mobilization of EPC. Although a formal correlation between tumor type/stage/size and number of EPC has not been established in human cancer, some tumor types may be more dependent than others on CEC as a source of endothelium [92].

Taken together, the results of these studies indicate that the contribution of EPC to neovascularization is not restricted to normal healing processes and that they contribute significantly to several pathological processes. One of the most intensively studied models of EPC and neovascularization is tumor angiogenesis, as described subsequently.

MESENCHYMAL STEM/MARROW STROMA CELLS

An especially critical BM-derived stem/progenitor cell is the MSC. MSC reside in the perivascular regions of the hematopoietic niche of the BM, from which they provide a wide array of physical and chemical signals that are required to drive or regulate the hematopoietic process. MSC or MSC-like cells also exist in close association with the vasculature throughout the rest of the body as so-called pericytes. MSC possess a set of unique properties that combine to make them ideally suited for cellular therapies and regenerative medicine, and as vehicles for gene and drug delivery. Much research has been focused on finding ways to mobilize endogenous MSC from the BM and/or from the widely distributed pericyte reserves or to administer exogenous MSC to mediate tissue repair and thereby treat a variety of injuries and diseases. Some important properties that make MSC one of the most promising stem cell populations for regenerative medicine and vehicles for gene delivery (to be covered in a later section) include (1) their relative ease of isolation; (2) the ability to differentiate into a wide variety of functional cell types of both mesenchymal and nonmesenchymal origin in vitro and in vivo; (3) the ability to be expanded extensively in culture without a loss of differentiative capacity; (4) the fact that they are not only hypoimmunogenic but produce immunosuppression upon transplantation; (5) their pronounced antiinflammatory properties; and (6) their ability to home selectively to damaged tissues after in vivo administration, while contributing little if anything to normal or healthy tissue [1,93,94]. In the section that follows, we will briefly discuss the discovery of MSC, their basic biology and characterization, and their ability to be expanded in vitro for therapeutic use.

Identification, Isolation, Characterization, and In Vitro Expansion

In pioneering studies [95] performed nearly 4 decades ago, Alexander Friedenstein demonstrated that fibroblast-like cells obtained from the BM were able to transfer the hematopoietic microenvironment to ectopic sites, thus establishing the concept that the marrow microenvironment resided within the so-called stromal cells of the BM. However, it was the pioneering work of Caplan that established the concept of the mesengenic process [96] and led to the realization that this population harbored cells with properties of true stem cells, termed MSC [96]. MSC are now known to make up a key part of the stromal microenvironment that supports the HSC and governs the process of hematopoiesis. Despite their essential role, MSC are exceedingly rare within the BM; they account for only 0.001%−0.01% of the total marrow cellularity [97].

Like many rare stem cell populations, MSC cannot be isolated to absolute purity, but numerous culture methods and purification procedures have been developed by various laboratories. The most straightforward method for obtaining MSC is simply to rely on the plastic adherence of MSC and their ability to be passaged with trypsin, to obtain a relatively morphologically homogeneous population of fibroblastic cells from a bulk MNC preparation after only two to three culture passages. This method is certainly straightforward, but true MSC account for only a small percentage of this highly heterogeneous population, which can greatly complicate the interpretation of experimental results obtained with cells prepared in this manner. To avoid this problem, numerous groups have worked for years to identify antigens that are unique to MSC. Interestingly, human MSC do not express markers associated with other stem cell populations (such as HSC), such as CD34, CD133, or c-kit. Indeed, no markers known to identify MSC uniquely. However, several markers have proven useful for obtaining cell populations that are enriched for MSC. The first such marker to be identified was Stro-1 [98]. Although the antigen recognized by the Stro-1 antibody has not yet been identified, we and others have shown that by tri-abeling BM cells with Stro-1, anti-CD45, and antiglycophorin A (GlyA) and selecting the $Stro^{-}1^{+}CD45^{-}GlyA^{-}$ cells, it is possible to obtain a homogeneous population consistently that is highly enriched for MSC [99,100]. In addition to Stro-1, other antibodies such as SB-10, SH2, SH3, and SH4 have been developed over the years, and numerous surface antigens such as CD13, CD29, CD44, CD63, CD73, CD90, and CD166 have been used to attempt to isolate MSC [101−103]. Unfortunately, all of these antigens appear to be expressed on a wide range of cell types within the body in addition to MSC. This lack of a unique marker suggests that to obtain a pure population of MSC that are functionally

homogeneous, investigators likely will either have to await the development of novel antibodies that recognize as yet unidentified antigens unique to primitive MSC or employ strategies in which multiple antibodies are combined, to allow for the positive selection of MSC and depletion of cells of other lineages that share expression of the antigens recognized by the MSC antibody in question, as we have done with Stro-1, CD45, and GlyA. To facilitate the comparison of results from different groups and encourage the use of a better-defined population of MSC in experimental and clinical studies, the International Society for Cellular Therapy stated that MSC must possess several characteristics aside from simple adherence to plastic. They must express CD105, CD73, and CD90, but not express CD45, CD34, CD14, CD11b, CD79, or CD19 and human leukocyte antigen (HLA)−D related surface markers. Furthermore, they must be able to differentiate into the three key MSC types found within the BM, i.e., bone, cartilage, and fat [104].

Although much has focused on MSC isolated from adult BM, cells that appear phenotypically and functionally to be MSC have been isolated by our group and others from numerous tissues including umbilical cord blood, cord matrix, amniotic fluid, placenta, brain, liver, lung, fetal blood, kidney, and even liposuction material [105−109]. The broad distribution of MSC throughout the body has led to the supposition that MSC are likely to have a critical role in organ homeostasis, perhaps providing supportive factors as in the BM, and/or mediating maintenance and repair within their respective tissue. Importantly, although MSC from each of these tissues appear similar with respect to phenotype and overall differentiative potential, studies at the RNA and protein levels revealed that subtle but important differences exist among MSC from these various tissues, with MSC from each tissue possessing a molecular fingerprint indicative of their tissue of origin [110,111]. Using a noninjury fetal sheep transplantation model, we showed that these differences result in a bias for human MSC to home to and develop into cells of their tissue of origin in vivo [112]. This suggests that the ideal source of MSC may differ depending on the specific disease to be treated and the desired target organ. Despite the apparent presence of MSC within many of the major organs of the body, however, the relatively noninvasive fashion with which adipose tissue or BM can be obtained, and the fact that both of these tissues could readily be obtained from each patient to be treated, suggest that these two tissues will likely be the predominant sources of MSC employed in clinical applications in the foreseeable future.

Although MSC constitute a small percentage of the nucleated cells present in the BM, they can be expanded exponentially while maintaining their original phenotype and differentiative potential [113]. Indeed, Bruder et al. [114] demonstrated that human BM-derived MSC can readily be propagated in vitro until passage 38 ± 4 before senescing. Moreover, by plating these cells at a low density ($\sim$1500−3000 cells/cm^2) and consistently passaging them before they reach confluence, it is possible to accelerate their growth rate, increase their expansion capacity, and avoid the spontaneous differentiation that often occurs at higher cell densities [115]. This ability to be expanded extensively in vitro has two important implications for their use in cellular-based liver therapies. The first of these is that a small BM aspirate could be taken from the patient and adequate cells may be obtained for transplantation through extensive expansion in vitro after isolation. Second, by virtue of their ability to be expanded in culture without loss of in vivo potential, MSC could be harvested from the patient's own marrow even if the disease to be treated is the result of an underlying genetic defect, because MSC can readily be genetically manipulated in vitro with both viral and nonviral vectors [1,94,116]. This will be discussed in detail subsequently. After genetic manipulation, a pure population of genetically corrected autologous MSC could then be propagated to generate sufficient numbers of cells to achieve meaningful levels of engraftment after transplantation.

However, additional in vitro and in vivo experiments will likely have to be performed to assess the inherent safety of the specific in vitro−expanded MSC in question rigorously before their clinical use, because at least one study suggested that MSC from adipose tissue may be inherently less genetically stable than MSC isolated from BM [117]. Indeed, two other studies showed that upon prolonged in vitro expansion, adipose tissue−derived MSC can exhibit aneuploidy [118] and have been shown to undergo transformation [119], which raises the possibility that these cells could prove tumorigenic when used in vivo. In stark contrast, however, are the results of another study investigating the inherent safety of adipose-derived MSC. That study showed that even if genomic instability is intentionally induced with genotoxic agents, adipose tissue−derived MSC respond to this insult by undergoing terminal adipogenic differentiation rather than transformation [120]. The reasons for the conflicting nature of the results from these different studies are not known, but one can speculate that differing methods employed for isolating and culturing MSC, differing levels of contaminating non-MSC cells in the cultures, as well as the duration of the culture (i.e., the number of times the cells were passaged) were likely contributing factors. With this possible instability in mind, at this point it seems prudent, regardless of the tissue source of the MSC, to make clinical use only of cells that have been passaged fewer than 25 times in culture [121].

THERAPEUTIC APPLICATIONS OF PERIPHERAL BLOOD STEM CELLS

The physiological role of MSC in tissue regeneration prompted researchers to evaluate their use in therapeutic applications. Ethical discussions regarding embryonic stem cells underscore the need to explore the clinical applications of adult stem cells, including MSC. MSC were first tested in several animal models and have been used in multiple clinical studies. Although the results are promising, the mechanisms behind the regenerative potential of MSC are not fully understood. Their therapeutic applications can be divided into three groups: tissue engineering, cell delivery applications, and as a vehicle for gene therapy [1,46,94].

Endothelial Progenitor Cells

Angiogenesis and Vasculogenesis

Blood vessels are constructed by two processes: angiogenesis, or the sprouting of capillaries from preexisting blood vessels and vasculogenesis; and the in situ assembly of capillaries from undifferentiated EC. Formation of the first capillaries takes place mostly during the early stages of embryogenesis [72]. Early vascular plexus in the yolk sac originates from the mesoderm by differentiation of angioblasts, which subsequently generate primitive blood vessels. Angiogenesis is a morphogenic process of new blood capillaries emerging from preexisting vessels. Angiogenesis has a major role in wound healing and collateral formation and in the female reproductive system. In wound healing and tissue repair, neovascularization is required to transport cells and nutrient into the wound. The new blood capillaries emerge from neighboring vessels during tissue remodeling. In the female reproduction cycle, neovascularization occurs physiologically in the uterus and ovary every 28 days. Placental tissues represent a major site for active angiogenesis [122]. Myocardial collateral vessels that sprout from preexisting capillaries are important to protect the heart from ischemic damage. In myocardial infarction, new vessels penetrate the necrotic area and the surrounding ischemic heart tissues [123]. Many serious diseases are associated with excess capillary growth. Most notably, angiogenesis is associated with cancer, tumor growth, and metastasis [124]. Intraocular neovascularization is usually associated with diabetic retinopathy and retinopathy of prematurity [125]. The new blood vessels are leaky and easily rupture, which may result in blindness. In chronic inflammatory diseases such as rheumatoid arthritis, new vessels invade the joint surfaces and degrade the cartilage by proteolysis [126]. The vasculogenesis process can be divided into five consecutive steps [127]: (1) EC are generated from precursor cells called angioblasts. (2) EC form the vessel primordia and aggregates that establish cell-to-cell contact but have no lumen. (3) A nascent endothelial tube is formed composed of polarized EC. (4) A primary vascular network is formed from an array of nascent endothelial tubes. (5) Pericytes and vascular SMC are recruited. Circulating EC originating from endothelial EPC in the BM are mobilized into the bloodstream and participate in angiogenic processes [66,89]. Based on the role of EPC in postnatal neovascularization [66], attempts were made to use EPC for therapy as a means to support tissue regeneration.

EPC can be used for therapeutic vascularization in two ways: by mobilizing of EPC into the circulation using growth factors and by first expanding them in vitro followed by systemic delivery or direct injection into the target tissue or organ [128,129]. Although EPC were shown to contribute to tissue revascularization, their function in a clinical setting remains controversial [130]. On the other hand, there have been significant advances in the use of EPC for tissue engineering. Several examples are provided subsequently of the use of EPCs for tissue regeneration and vascularization and as the endothelial source for bioengineered tissues and organs.

Tissue Regeneration

In many cases, organ and tissue regeneration require reestablishment of the vascular network. During healthy and pathologic neovascularization, reendothelialization can occur through mature EC that migrate from preexisting vessels via angiogenesis [131] and circulating EPC from PB that integrate with newly formed vasculature via vasculogenesis [47,128,132]. EPC can be obtained from the patient and expanded in culture to avoid immune rejection; they offer a robust cell source for tissue regenerations.

Several studies suggested that EPC participate in the vascular healing process, in part by recruiting EPC to the regenerated site [40,133]. Genetically labeled EPC were detected in ischemic limbs of mice and were shown to accelerate the revascularization process. In humans, EPC contributed to wound healing of patients implanted with a left ventricular assisted device [41]. The EPC adhered to the device and formed a nonthrombogenic surface. These studies suggested that EPC may be recruited to assist endothelialization and served as the basis for preclinical and clinical studies, as described subsequently. Administration of cytokines including G-CSF, GM-CSF, and

angiogenic growth factors appear to enhance the mobilization of EPC and revascularization. These factors are usually released into the circulation as a result of injuries [128]. Based on these observations, PB progenitors such as EPC were used for cell therapy to combat tissue ischemia and support tissue regeneration. Main clinical targets for the cell therapy studies were CVD, injuries in the brain, and retinal ischemia [129]. Animal studies showed improved vascularization and tissue function.

Systemic delivery of VEGF protein, one of the most potent angiogenic growth factors in vivo, and VEGF gene therapy resulted in a significant increase in EPC numbers in the circulation and their contribution to neovascularization [40]. Elevated Epo levels in the circulation were shown to increase the numbers of VEGFR-2−positive EPC [54]. In parallel, data from Epo-derived cell mobilization in humans demonstrated an increase in $CD34^{+}/CD45^{-}$ cells, probably circulating EC [134]. Gene delivery of the hematopoietic growth factor CXCL12 (SDF) also led to an increase in VEGFR-2−positive EPC [135]. Interestingly, AMD3100, the SDF receptor binding inhibitor, caused EPC mobilization to the peripheral circulation.

Melero-martin et al. [62] reported that when cord blood EPC and MSC were injected subcutaneously into nude mice, they formed vascular structures that were stable for 4 weeks. The vascular network had endothelialized lumen and pericytes expressing smooth muscle actin. Au et al. [136] engineered blood vessels from HUVEC and human MSC that remained stable and functional for more than 130 days in vivo. These findings could guide future practices in tissue engineering and regenerative medicine, allowing physicians to form stable and long-lasting vasculature for engineered tissue.

CVD are an attractive target for EPC-mediated tissue neovascularization as a means of improving regeneration and function. Accordingly, several promising animal studies led to clinical trials involving patients who had experienced acute myocardial infarction (AMI) and cardiac ischemia. BM-derived MSC were shown to contribute to myocardial regeneration and revascularization. In nude rats that underwent myocardial infarction, cytokine-mobilized EPC homed to the infarcted tissue and contributed to neoangiogenesis [137]. In similar studies, BM-derived MSC were injected into the infarcted border and were shown to differentiate into myocardial cells and EC [137,138]. In most studies, direct introduction of these cells into an active angiogenic site, such as infarcted or ischemic myocardium, was essential for the successful incorporation of the cells and improvement in cardiac function.

AMI or chronic ischemic heart disease results in the loss of cardiomyocytes and vasculature. Several animal studies showed that the introduction of autologous BM MSC contributes to neoangiogenesis in the ischemic myocardium [46]. In patients, whole autologous BM MNC were delivered into the coronary arteries feeding the infarcted and ischemic tissue [46]. In all of these studies, there was improved cardiac perfusion and left ventricular function, which suggested that delivery of autologous progenitor cells is feasible and safe and may have a short-term therapeutic benefit. However, follow-up studies in animals and humans detected only a few BM-derived cells in the regenerated vascular network, which suggested that only a small portion of the cells may contribute to revascularization.

Delivery of autologous MNC or G-CSF−mobilized MNC showed some improvement in left ventricle ejection fraction, especially when larger numbers of cells were infused directly into the heart [139−141]. However, no long-term information is available on the clinical outcomes of the patients. Other studies of clinical intervention with cell therapy for AMI showed some clinical benefits [142]. Yet, some adverse events such as restenosis and an increased risk for atherosclerosis were also reported after cell therapy with PB-derived cells [128]. Altogether, despite early promising results from animal studies and short-term data from clinical studies of cell therapy for CVD, there is a lack of long-term outcome assessment of clinical trials that would define the patient population most likely to benefit from the treatment of cell therapy. It remains unclear whether any long-term toxicity exists with this therapy. Such toxicity may result if myeloid cells are incorporated into regenerating myocardium and generate noncardiac or fibrotic tissues.

Given the morbidity associated with limb ischemia, EPC may be used for vascular therapy as an alternative to bypass approaches. In preclinical studies, the introduction of BM-derived EPC significantly improved collateral vessel formation and minimized limb ischemia [143]. In patients with peripheral arterial disease, injection of autologous whole BM MNC into ischemic gastrocnemius muscle resulted in the restoration of limb function [144]. The improvement in muscle perfusion suggested that it was caused by the presence of EPC in the cell preparation. However, it remains to be determined whether the improvement resulted in part from the introduction of myelomonocytic cells.

Diabetes is often associated with CVDs, which can be treated with systemic and directed injections of PB-borne EPC, as described earlier. In addition, EPC can directly support the regeneration of pancreatic tissue. Intraportal transplantation of pancreatic islets covered with EPC improved the survival of grafted islets and their function in

immune-deficient mice [129]. It appears that the islet-associated EPC remained at the implantation site and supported β-cell proliferation and insulin secretion. Subcutaneous implantation of EPC combined with porcine pancreatic islets in collagen/fibronectin gels resulted in a functional vascular network after 2 weeks [145], which suggests that EPC can be combined with other cell therapy applications to support reestablishment of the vasculature at the site of cell injection.

Pulmonary diseases are progressive lung disorders often manifested by smaller arteries associated with intimal hyperplasia and fibrosis, increased deposition of ECM, and prolonged inflammation, which may lead to respiratory complications [146]. Cellular therapies for pulmonary-associated diseases employed progenitor cells of different types that aimed to reduce the symptoms and potentially enhance regeneration of the diseased lung tissue. EPC were used for cell therapy in rats treated with monocrotaline (MCT) to create pulmonary arterial hypertension (PAH) [147]. When delivered 3 days after MCT treatment, they effectively prevented PAH. However, once a state of PAH was established after 3 weeks, the cells had only marginal effectiveness, but they prevented further worsening of symptoms. On the other hand, delivery of EPC recombinantly expressing eNOS significantly reduced right ventricle systemic pressures [147]. Based on these promising results, a phase I clinical trial was initiated to test the tolerability of eNOS-expressing autologous EPC in patients with severe PAH [147].

Tissue Engineering

Vascular diseases are the leading causes of morbidity and mortality in the United States each year [148]. Over 500,000 coronary bypass grafts and 50,000 peripheral bypass grafts are performed annually in the United States (www.americanheart.org) [149]. However, up to 30% of patients who require arterial bypass surgery lack suitable or sufficient amounts of suitable autologous conduits such as small-caliber arteries or saphenous veins [150]. Synthetic grafts such as polytetrafluoroethylene or Dacron (polyethylene terephthalate fiber) have been used successfully to bypass large-caliber, high-flow blood vessels. However, these grafts invariably fail when used to bypass small-caliber, low-flow blood vessels owing to increased thrombogenicity and accelerated intimal thickening leading to early graft stenosis and occlusion [151]. It has been shown that a confluent EC monolayer on small-caliber prosthetic grafts may provide immediate protection from thrombus formation after implantation [152]. However, the use of allogeneic EC is limited by rejection, whereas the use of autologous human EC to construct vascular grafts has not been widely explored. The idea of using EPC to seed the lumen of engineered blood vessels came from the observation that MSC contributed to the lining of vascular grafts in vivo [129,153,154]. We have shown that EPC might be an ideal source of autologous EC for seeding small-diameter grafts, which eliminates the need to remove native vessel from which to culture EC. By seeding EPC-derived EC onto a scaffold, a nonthrombogenic barrier between blood and the vessel wall is created, thereby promoting patency in vivo. EPC-seeded collagen matrices derived from decellularized porcine arteries were used for carotid artery reconstruction in sheep [155]. These bioengineered arteries remained patent more than 4 months, whereas control grafts without autologous EC occluded within 15 days. Thus, functional vessels can be engineered using decellularized arteries and EPC. Moreover, we have shown that after a brief period of healing in vivo, these bioengineered blood vessels develop a fully cellularized wall of three distinct layers analogous to normal adventitia, media, and intima. A similar approach was used to coat vascular stents with EPC to minimize neointimal cell growth and reduce the incidence of restenosis [156]. Yoder and colleagues used mobilized progenitor cells to coat left ventricular assist devices [157]. Although these are exciting results, bioengineered grafts and vascular stents will need to be preconditioned to blood flow before implantation. Neram et al. [158] showed that local blood flow properties induce changes in endothelial cell morphology and orientation. Further studies showed that the levels of shear stress and duration of exposure induced changes in EC morphology, proliferation, and differentiation.

EPC were used in several studies to coat the surface of bioengineered heart valves. Two endothelial cell types were used: valve-derived mature EC and EPC from PB [159]. The study showed that when seeded on polyglycolide/poly-4-hydroxybutyrate (PGA/P4HB) scaffolds, both sources of EC proliferate in response to VEGF. The EPC could be induced to transdifferentiate to a mesenchymal phenotype on PGA/P4HB in response to transforming growth factor-β1. These results indicate that EPC can respond to soluble signals that induce events that occur during valvulogenesis. Another approach to coating the valve surface with an endothelium layer was described by Jordan and Williams and colleagues [160,161]. Those authors used decellularized porcine pulmonary valves that were conjugated with CD133 antibodies on the leaflet surface. The valves were transplanted into the pulmonary position of sheep and harvested for analysis at different times up to 3 months. $CD133^+/CD31^-$ EPC adhered to the surface of anti-CD133–conjugated valves by 3 days and transitioned to $CD133^-/CD31^+$ mature EC by 30 days. ECs expressing von Willebrand factor lined the entire length of both ventricular and arterial surfaces of conjugated valves by 1 month after implantation. Interstitial cells expressing both α-smooth muscle actin and vimentin were observed

as early as 14 days postimplantation and their content increased from 1 to 3 months in valves conjugated with anti-CD133, with new collagen formation. In contrast, only a few EC or interstitial cells were associated with unconjugated valves at these times.

A new scaffolding technology for tissue and organ regeneration was introduced to the field. The technique is based on the decellularization of intact human and animal organs by infusing the organ's vasculature with mild detergents that completely remove the cellular components while not degrading the ECM [162]. We successfully made such acellular whole-organ scaffolds for liver, kidney, pancreas, and intestine. The decellularization process preserved an intact network of vascular channels that can be used to seed cells into the scaffold and later to infuse culture media to deliver oxygen and nutrients to cells [163] to ensure the successful implantation of these scaffolds into a recipient that must resist thrombosis caused by exposed ECM in contact with blood. We seeded both acellular porcine kidneys livers with EC and showed vascular patency upon implantation in vivo. These studies used an EC line that cannot be used for clinical applications. Instead, collection of mobilized EPC from PB and propagation of the cells in culture represent a logical path for the revascularization of bioengineered organs for clinical use in humans. To improve the reestablishment of functional vascular network further, EPC can be combined with other cell types such as mural cells. Combining EPC with pericyte progenitors such as MSC or with SMC or muscle progenitors significantly improves the stability and preserves functional blood vessels and subsequently tissue and organ function [129]. EPC were also used to support the bioengineering of bone. EPC combined with MSC in collagen/fibronectin gel were seeded into decalcified cancellous bone for calvarial defect repair [164]. Only in the presence of EPC did host blood vessels penetrate into the implant and induce bone formation.

Wound healing is a major problem that affects all aspect medical treatments. Studies over the past 5 decades clearly showed that wound healing greatly depends on the successful neovascularization of the injured tissue [165]. Tissue engineering technologies combining biomaterials and cells can provide both important coverage of the wound and improve wound healing. For example, silk-based scaffolds can be populated with EPC and used as a skin construct [166]. By adding other cell types to the scaffold, such as pericyte progenitors and keratinocytes, the construct can be used for skin transplantation. In other applications, EPC were combined with dermal fibroblasts to bioengineer skin substitutes [167,168]. The resulting skin constructs were well-perfused and showed adequate epithelialization and normal matrix deposition.

EPC represent an ideal cell source for tissue engineering because they are easily obtained from the patient and expanded in culture and are functional in the bioengineered tissue. However, in several cases, heterogeneous cell populations are being expanded for seeding onto scaffolds, which may lead to compromised function as a result of differential proliferation of cells with fibroblastic properties. To overcome this, one may use specific methods to obtain a pure EPC population before seeding, such as immune isolation. An additional problem is the occasional need for immediate availability. For instance, when an emergency bypass needs to be performed, the growth of a bioengineered blood vessel ready for implantation would take too much time if autologous cells are to be implemented. Alternatively, these bioengineered grafts could be seeded with autologous MSC cells that are differentiated into EC using a shorter protocol.

Mesenchymal Stem Cells

The ability of MSC to differentiate in vitro and in vivo into the various mesenchymal cell types found within the BM (i.e., bone, cartilage, and fat) has been described by numerous laboratories, and the conditions for bringing each of these differentiative pathways about have been delineated in detail [169]. Microarray studies [170] have shed light on the molecular mechanisms responsible for commitment to and progression along each of these lineages, providing information needed to enhance the efficiency with which MSC differentiate to each of these lineages, revealing some of the pathways that maintain MSC in an undifferentiated state, and enabling more robust in vitro expansion of this valuable stem cell population. Promising results from preclinical studies examining the use of MSC for bone and cartilage repair [171] quickly led to clinical trials using MSC to treat large bone defects [172], articular cartilage defects [173], and osteogenesis imperfecta [174]; these trials have highlighted the therapeutic potential of MSC.

Although these early studies quickly established MSC as a promising therapy for treating a wide range of skeletal injuries and diseases, further investigation over the past several years has provided compelling evidence that the differentiative capacity of MSC far exceeds that originally reported by Friedenstein. For example, MSC also have the ability to generate functional skeletal muscle cells in vitro and in vivo [175,176], which raises the possibility that MSC may eventually be used to treat muscular dystrophies. Even more surprising were subsequent studies showing that MSC had the ability to develop not only into cells of mesenchymal derivation, but into cells of all three

germinal layers. The first dogma-breaking study demonstrating that MSC had the ability to cross developmental boundaries and reprogram into cells of another germ layer demonstrated that MSC could become both neural and glial (ectoderm) cell types in vitro and in vivo [177–179]. These findings raised the exciting possibility that MSC could serve as a readily available source of cells to treat injuries or degenerative conditions within the central nervous system (CNS), clinical situations for which there are limited options, if any. Indeed, numerous in vivo transplantation studies have confirmed these exciting in vitro results by showing that MSC have the ability to mediate repair after spinal cord injury, ischemic injury and stroke, demyelinating conditions, and experimentally induced Parkinson disease, and potentially treat a host of other injuries and degenerative diseases of the CNS [180]. Although questions have been raised as to the mechanism by which this apparent "transdifferentiation" occurs [181], the demonstration by numerous groups that MSC can indeed develop into neural cells in various stages of differentiation and mediate functional improvement for a variety of injuries and diseases within the CNS provides the impetus for further studies attempting to increase the efficiency and predictability of this process and harness this potential for therapy.

After these groundbreaking findings, studies were published showing that MSC could develop in vitro into cells that appear phenotypically and morphologically like cardiomyocytes, and behave like them [182,183] and to endothelium [184,185], and to engraft within the injured myocardium [55,186–189] and form Purkinje fibers in vivo [190,191]. These preclinical studies generated tremendous excitement in the field of regenerative medicine, because they strongly suggested that MSC could be used as therapy to mediate cardiac repair postinfarction or diseases within the conduction pathways of the heart, or even, perhaps, to treat chronic progressive cardiac diseases such as congestive heart failure [192]. Coupled with preclinical animal studies showing functional cardiac improvement after infusion of MSC, or even MSC-conditioned media [193–195], these exciting results were the driving force for launching several clinical trials to investigate whether MSC are able to mediate repair in the postinfarct heart [196–198].

As a last major example of MSC in regenerative medicine, we will address their use in liver repair. Liver failure is a life-threatening condition for which the only curative treatment is whole or partial organ transplantation. Given the crippling shortage of donor livers available for transplantation and the high mortality and morbidity associated with the need for lifelong immunosuppression after liver transplantation, a major focus of regenerative medicine has been to identify cells that could be used to repopulate and/or repair the damaged failing liver. We and others have devoted a great deal of energy to demonstrating the ability of MSC from various sources to serve as therapeutics for liver disease [199,200]. It is clear that MSC isolated from a variety of mouse, rat, and human tissues have the ability to generate in vitro and in vivo cells that are indistinguishable from native hepatocytes, in that they exhibit the same morphology and antigenic profile as native hepatocytes, and they appear to be functional, based on the uptake of low-density lipoprotein, urea production, and glycogen storage. Whether these cells exhibit polarity like native hepatocytes has yet to be unequivocally demonstrated, however. Moreover, transplantation of MSC in a range of model systems has repeatedly been shown to result in the fairly robust formation of hepatocytes that repair a variety of inborn genetic defects, as well as mechanical and drug or toxin-induced injuries, including partial hepatectomy, treatment with the toxin C-C motif chemokine ligand 4, injury induced by allyl-alcohol, a high-lipid diet, treatment with 2-acetylaminofluorene, and even pathogenic infection [200]. This wealth of supportive data in several different species has led to the use of MSC for liver disease in multiple clinical trials [200]. Collectively, these clinical studies have provided hope that MSC may prove to be a valuable resource for cell-based therapies for liver disease. However, the results of these studies must be interpreted with trepidation because of the limited number of patients enrolled in each trial, the lack of appropriate controls in many cases, and the occurrence of adverse events. Furthermore, because the cells in these trials were derived autologously, there was no way for the investigators to assess the actual engraftment, persistence, or differentiative potential of the transplanted cells, which leaves the mechanism responsible for the observed clinical improvements open to speculation.

A key aspect to assessing the utility of MSC therapy for regenerative medicine is the mechanism by which the transplanted cells repair, replace, or repopulate the recipient tissue(s). Indeed, there has been much controversy regarding the mechanism by which MSC reprogram and differentiate into other cell lineages, such as hepatocytes. Whereas the previously summarized in vitro studies unequivocally showed that under the appropriate conditions, MSC can reprogram to generate what appear to be functional hepatocytes, it appears that in vivo, cell fusion could be one mechanism by which MSC appear to develop into hepatocytes, rather than true reprogramming or transdifferentiation [79,201,202]. To begin delineating the mechanism(s) of hepatocyte formation after transplantation of human MSC, we performed studies in the fetal sheep model [100], which we believed would be ideal for this analysis, given the robust generation of human-derived hepatocytes. By using carboxyfluorescein succinimidyl

ester- or DiIC18(3)-labeled human MSC and performing fluorescence in situ hybridization on liver sections from sheep transplanted in utero with these labeled human MSC, we were able to demonstrate conclusively that transplanted human MSC were able to generate significant numbers of functional hepatocytes in the absence of cellular fusion or membrane vesicle or organelle transfer [99,100] independent of fusion, and by true reprogramming or transdifferentiation [100]; this suggests that these cells indeed possess a degree of true plasticity. The lack of fusion as a requirement for liver repopulation was in stark contrast to other studies in which the transplanted stem cells' differentiative capacity was restricted to cells of the liver through drug- or genetically induced lesions or deficiencies [79,201,202], and suggests that the mechanism by which the transplanted MSC contribute to the recipient liver (and likely other tissues) depends on the model system employed.

The interpretation of data generated from these myriad studies looking at the potential of MSC to mediate repair in a variety of organ systems is further complicated by the observation that a therapeutic benefit is often observed in the absence of evidence of long-term engraftment of the transplanted MSC within the damaged or diseased organ. These findings led to much debate regarding whether MSC can actually generate tissue-specific cells after transplantation, and sparked additional studies showing that MSC can mediate tissue repair by acting as "trophic factories," releasing specific cytokines, growth factors, and matrix MMP that modulate the activity of tissue-specific cells, dampen ongoing inflammation and/or aberrant immune reaction present within the damaged or diseased tissue, and inhibit fibrosis and apoptosis, thus facilitating endogenous tissue regeneration [1,93,94,200]. However, under different conditions, other studies showed that transplanted MSC may actually contribute to the myofibroblast pool, enhancing the fibrotic process [203–205]. This has led to the current belief within the field that the effect of MSC will probably vary with the nature of the injury or disease that is being treated, the specific experimental model with which the therapy is being tested, and perhaps even the time frame of MSC application, such that MSC could be beneficial if administered at certain stages of disease progression and harmful if administered at others. Thus, it appears that the therapeutic potential of MSC may have to be investigated for each specific disease or injury to be treated, to delineate the optimal time frame and population to be administered to achieve the desired effect and ensure benefit is provided rather than harm.

The Use of Mesenchymal Stem/Marrow Stroma Cells for Gene Therapy

Although MSC possess tremendous therapeutic potential by virtue of their ability to lodge or engraft within multiple tissues in the body and both develop into tissue-specific cells and release trophic factors that trigger the tissue's own endogenous repair pathways, gene therapists have realized that these properties are just the beginning of the therapeutic applications for MSC [1,94]. By using gene therapy to engineer MSC to either augment their own natural production of specific desired proteins or enable MSC to express proteins outside their native repertoire, it is possible to broaden the spectrum of diseases greatly for which MSC could provide therapeutic benefit. Unlike HSC, which are notoriously difficult to modify with most viral vectors while preserving their in vivo potential, MSC can be readily transduced with all of the major clinically prevalent viral vector systems (adenovirus [206], the murine retroviruses [207], lentiviruses [208], and adeno-associated virus [209]), and efficiently produce a wide range of cytoplasmic, membrane-bound, and secreted protein products. This ease of transduction, coupled with the ability subsequently to select and expand only the gene-modified cells in vitro to generate adequate numbers for transplantation, combine to make MSC one of the most promising stem cell populations for use in gene therapy studies and trials.

Most studies using gene-modified MSC have been undertaken with the purpose of enhancing the natural abilities of MSC to mediate repair within various tissues. Using the heart as an example, once investigators discovered the identity of some of the most important trophic factors responsible for the beneficial effect of MSC on the injured myocardium, they undertook studies using MSC engineered to overexpress a number of factors [210,211]. As anticipated, the "gene-enhanced" MSC were substantially more effective than their unmodified counterparts and produced greatly enhanced therapeutic effects. Similar studies were performed to repair the damaged or diseased CNS using MSC engineered to produce neurotrophic factors [180], repair the injured liver using MSC-expressing proteins involved in hepatocyte differentiation and/or proliferation [212,213], repair ischemia-reperfusion injury [214], and repair the kidney [215]. In each case, MSC engineered to express higher levels of proteins known to be beneficial for the tissue in question and/or to promote survival have produced markedly better results than unmodified MSC.

Despite the many advantages of using MSC as gene delivery vehicles, however, relatively few studies have explored this potential to treat genetic diseases. One disease for which we and others are actively investigating MSC to deliver a therapeutic gene is hemophilia A (HA) [216–218]. We will use HA in the following discussion

as a paradigmatic disease to illustrate how gene-modified MSC could be used to correct essentially any genetic disorder that involves the absence of a specific protein.

HA is the most common inheritable deficiency of the coagulation proteins [219]. The severity of HA is traditionally based on plasma levels of factor VIII (FVIII) [220], and up to 70% of patients with HA present with the severe form of the disease (<1% of normal FVIII levels) and have frequent hemorrhaging leading to chronic debilitating arthropathies, hematomas of subcutaneous connective tissue or muscle, and internal bleeding. Over time, the collective complications of recurrent hemorrhaging result in chronic pain, absences from school and work, and permanent disability [220]. Current state-of-the-art treatment consists of frequent prophylactic infusions of plasma-derived or recombinant FVIII protein to maintain hemostasis. Although this treatment has greatly increased life expectancy and quality of life for many patients with HA, it is far from ideal because of the need for lifelong intravenous infusions and the high treatment cost, which often approaches $500,000/year. Moreover, this treatment is unavailable to a large percentage of the world's hemophiliac patients, which places them at great risk for severe, permanent disabilities and life-threatening bleeds. There is thus a significant need to develop novel, longer-lasting HA therapies.

In contrast to current protein-based therapeutics, lifelong improvement or permanent cure of HA is theoretically possible after only a single gene therapy treatment; indeed, several aspects of HA make it ideally suited for correction by gene therapy. First, in contrast to many other genetic diseases, the missing protein (FVIII) does not need to be expressed in either a cell- or tissue-specific fashion to mediate correction. Although the liver is thought to be the natural site of FVIII synthesis within the body, as long as FVIII is expressed in cells that have access to the circulation, it can be secreted into the bloodstream and exert its appropriate clotting activity. Second, levels of FVIII-expressing cells even as low as 3%−5% would be expected to convert severe HA to a moderate or mild phenotype, reducing or eliminating episodes of spontaneous bleeding and greatly improving quality of life [221]. Based on this knowledge, the American Society of Gene and Cell Therapy (www.ASGCT.org) provided National Institutes of Health Director Dr. Francis Collins with a road map of disease indications that it believed would be viable gene therapy products. The patients with hemophilia were identified as being in the most promising "Target 10" group of diseases.

As mentioned in the preceding section, the liver is thought to be the primary site of FVIII synthesis within the body. As discussed in previously we and others have devoted much energy to demonstrating the ability of MSC from various sources to serve as therapeutics for liver disease. In ongoing studies, we found that after transplantation into fetal sheep, human MSC engraft up to 12% within the recipient liver [99,100] and contribute to both the parenchyma and the perivascular zones of the engrafted organs, which makes them ideal for delivering FVIII into the circulation. Because FVIII levels of 3%−5% of normal would convert a patient with severe HA to a moderate or mild phenotype, these levels of engraftment should be highly therapeutic. Thus, these collective results suggest that MSC may represent an ideal cell type for treating HA.

However, although MSC engrafted (after transplantation in utero) at significant levels within organs that are natural sites of FVIII synthesis, only a small percentage expressed endogenous FVIII, which suggests that simply transplanting "healthy" MSC will not likely provide an effective means of treating or curing HA. By using gene therapy to engineer MSC to express FVIII, however, it is highly probable that the levels of engrafted MSC we have achieved thus far in utero would provide a marked therapeutic benefit in HA. By transducing the MSC in vitro, rather than performing gene therapy by injecting the vector directly, as is the current practice in clinical gene therapy trials for hemophilia B [222], there is no risk of off-target transduction, and the vector being employed simply needs a strong constitutively active promoter to ensure that all cells derived from the transplanted MSC continue to express FVIII and mediate a therapeutic effect. The only documented cases of viral vector-induced insertional mutagenesis were observed after genetic modification of HSC [223]; there is no evidence that MSC transform or progress to clonal dominance after transduction, which suggests that they represent safe cellular vehicles for delivering FVIII (or other transgenes).

Critical proof-of-principle studies have shown that MSC can be transduced with FVIII-expressing viral vectors and secrete high levels of FVIII protein in vitro and after transplantation in vivo [216−218]. FVIII purified from the conditioned medium of the transduced MSC was proven to have a specific activity, relative electrophoretic mobility, and proteolytic activation pattern that was virtually identical to that of FVIII produced by other commercial cell lines [217]. Given the widespread distribution and engraftment of MSC after their systemic infusion, the ability of MSC to develop in vivo into cells of numerous tissue types, and their ability to process and secrete high amounts of biologically active FVIII efficiently, unsurprisingly, they are being viewed as ideal vehicles for delivering an FVIII transgene throughout the body and thus providing long-term or permanent correction of HA [216−218].

In addition to their widespread engraftment and ability to serve as delivery vehicles for the FVIII gene, the unique immunological properties of MSC may further increase their utility for treating HA. MSC do not normally express major histocompatibility complex (MHC) class II or the costimulatory molecules CD80 and CD82, and thus viewed

are as being relatively hypoimmunogenic. As such, they do not provoke the proliferation of allogeneic lymphocytes or serve as effective targets for lysis by cytotoxic T cells or natural killer cells. In fact, a large body of evidence has accumulated suggesting that MSC can be transplanted across allogeneic barriers without eliciting an immune response. Thus, one could theoretically use off-the-shelf MSC from an unrelated donor to treat HA, which greatly increases the feasibility of obtaining and using these cells for therapy.

Perhaps even more important from the standpoint of their potential use as HA therapeutics, studies have demonstrated that MSC also have the ability to intervene at multiple levels with the generation and propagation of an immune response, and thus exert potent immunosuppressive and antiinflammatory properties both in vitro and in vivo [224,225]. In addition to interacting directly with cells of the immune system, MSC express a battery of factors that reduce local inflammation, blunt immune response, and counteract the chemotactic signals responsible for recruiting immune cells to sites of injury or inflammation. One could thus envision these immune-dampening properties enabling the delivery of FVIII without eliciting an immune response and subsequent inhibitor formation, thus overcoming the most serious clinical problem that plagues current treatment and management of HA [226].

In addition to these properties, preclinical animal studies examining the potential of MSC isolated from adult tissues have highlighted another interesting and clinically valuable characteristic of MSC: their ability to navigate to sites of injury and/or inflammation selectively within the body, as discussed in detail in the preceding section on MSC in regenerative medicine. Although the mechanisms responsible for this trafficking to sites of injury are still being elucidated, this observation raises the exciting possibility that after systemic infusion, FVIII-expressing MSC could migrate efficiently to sites of active bleeding or injury, releasing FVIII locally and focusing the therapy where it is needed most.

We have begun to explore whether it is possible to exploit these many advantages of MSC as a cellular vehicle to deliver an FVIII gene by testing the ability of FVIII-expressing MSC to correct HA in a unique line of sheep we reestablished and fully characterized, with respect to both the clinical parameters and the precise molecular basis for their disease [227]. Similar to mutations seen in many human patients, these animals possess a premature stop codon with a frameshift mutation. This is the only animal model of HA with this clinically relevant mutation type, which provides a unique opportunity to study therapies in this context. As with human patients with severe HA, a hallmark symptom in these sheep is repeated spontaneous joint bleeds that lead to chronic, debilitating arthropathies and reduced mobility.

To test the ability of MSC to serve as FVIII delivery vehicles and thus treat HA experimentally, we tested a novel, nonablative transplant-based gene therapy in two HA lambs [218]. During the first 3–5 months of life, both of these animals had received frequent, on-demand infusions of human FVIII for multiple hematomas and chronic, progressive, debilitating hemarthroses of the leg joints that had resulted in severe defects in posture and gait, rendering them nearly immobile. In an ideal situation, one would use autologous cells to deliver an FVIII transgene and thus avoid complications caused by MHC mismatching. Unfortunately, the severe life-threatening phenotype of the HA sheep prevented us from collecting BM aspirates to isolate autologous cells. We therefore elected to use paternal (haploidentical) MSC in the hope that immunologic incompatibility between the donor and recipient would be sufficiently minimized to allow engraftment.

Based on our prior work in the fetal sheep model, we knew that the intraperitoneal (IP) transplantation of MSC results in widespread engraftment throughout all of the major organs and durable expression of vector-encoded genes [99,100,228]. We further reasoned that using the IP route would have the advantage of enabling cells to enter the circulation in an almost time-released fashion after being engulfed by the omentum and absorbed through the peritoneal lymphatics. We also thought that the use of the IP route would enable us to avoid lung trapping, which hinders the efficient trafficking of MSC to desired target organs after intravenous administration and poses clinical risks owing to emboli formation [229,230].

After isolation, MSC were simultaneously transduced with 2 HIV-based lentivectors, the first of which encoded an expression/secretion optimized porcine FVIII transgene [218] that had previously been shown in human cells to be expressed or secreted at 10–100 times higher levels than human FVIII [231]. We thus believed that these high levels of expression and secretion might enable us to achieve a therapeutic benefit, even if we obtained low levels of engraftment of the transduced paternal MSC. The second lentivector encoded enhanced green fluorescent protein to facilitate the tracking and identification of donor cells in vivo. The first animal was then transplanted IP with the transduced MSC under ultrasound guidance in the absence of preconditioning.

After transplantation, this animal's clinical picture improved dramatically. All spontaneous bleeding events ceased and it had an event-free clinical course devoid of spontaneous bleeds, which enabling us to cease human FVIII infusions. Existing hemarthroses resolved, the animal's joints recovered fully and resumed normal

appearance, and it regained normal posture and gait and a normal activity level. To our knowledge, this represents the first report of phenotypic correction of severe HA in a large animal model after transplantation of cells engineered to produce FVIII, and the first time that reversal of chronic debilitating hemarthroses was achieved. However, despite this remarkable clinical improvement, FVIII activity (as assessed by chromogenic assay) was undetectable in the circulation of this animal after the MSC-based gene therapy treatment.

Based on the remarkable clinical improvement we had achieved in this first animal, we transplanted a second animal, increasing the dose of transduced cells in the hope of achieving detectable FVIII in the circulation. Similar to the first animal, hemarthroses present in the second one at the time of transplant resolved, and it resumed normal activity shortly after transplantation. The second animal also became factor-independent after the transplant. These results thus confirm the ability of this MSC-based approach to provide phenotypic correction in this large animal model of HA. However, as we had observed in the first animal, no FVIII was detectable in the circulation of the animal.

After the animals were killed, we performed a detailed tissue analysis to begin deciphering the mechanism by which this novel MSC-based gene delivery produced its pronounced therapeutic effect at a systemic level. Polymerase chain reaction analysis demonstrated readily detectable levels of MSC engraftment in nearly all tissues analyzed, including liver, lymph nodes, intestine, lung, kidney, omentum, and thymus. These molecular analyses thus proved that it is possible to achieve widespread durable engraftment of MSC after transplantation in a postnatal setting in a large animal model without the need for preconditioning or ablation, and in the absence of a selective advantage for the donor cells.

Confocal immunofluorescence analysis revealed large numbers of FVIII-expressing MSC within the synovium of joints that exhibited hemarthrosis at the time of transplant, demonstrating (as we had hoped and predicted) that the transplanted MSC possessed the intrinsic ability to home to and persist within sites of ongoing injury and inflammation, releasing FVIII locally within the joint. This explained the dramatic improvement we observed in the animal's joints. Confocal analysis also revealed engrafted cells within the small intestine, demonstrating that MSC could still engraft within the intestine after postnatal transplantation, as we observed in prior studies in fetal recipients [232]. Because of the ease with which proteins secreted from cells within the intestine can enter the circulation, future studies aimed at improving the levels of engraftment within the intestine have the potential to improve the systemic release of FVIII greatly.

The marked phenotypic improvement and improvement in quality of life we have observed in our studies in the sheep model thus support the further development of therapeutic strategies for HA and perhaps other coagulation disorders, employing MSC as cellular vehicles to deliver the required transgene. Extrapolating the work thus far in using MSC to deliver FVIII to treat HA, and combining this with the relative ease with which MSC can be isolated, propagated in culture, and modified with a variety of viral-based vectors, and their intrinsic ability to seek out sites of injury and inflammation within the body, one can readily see why MSC are widely viewed as being ideally suited not only as cellular therapeutics, but also as vehicles to deliver gene therapy vectors to numerous tissues in the body; thus, this is promising for finding a permanent cure for a diverse range of diseases.

CONCLUSIONS AND FUTURE DIRECTIONS

The BM is probably the source of PB stem and progenitor cells. Hemangioblasts are the embryonic precursors of HSC, developing into committed hematopoietic progenitors. The BM is also a source for other progenitor and stem cells, the MSC, which can be expanded in vitro and have multilineage differentiation potentials. Numerous studies, described earlier, showed that there is a constant exchange of cells from the BM to PB. These cells have shown proangiogenic properties and can be used for ischemic tissue neovascularization and tissue regeneration. The cells can be either mobilized using hematopoietic and angiogenic growth factors and recruited to the injured or regeneration tissues or they can be isolated, potentially expanded in culture, and delivered back to the same patient with or without a supporting scaffold system [128].

Future success in applying adult PB-derived stem cells for clinical applications will depend on the development of strategies to mobilize, isolate, expand, differentiate, and deliver these cells. For example, EPC may be isolated from PB and used for therapeutic angiogenesis directly or after a period of ex vivo expansion. Understanding the signals involved in recruiting these cells to the regenerating tissues will be crucial to optimizing this technology for clinical use. On the other hand, some concerns must be addressed before EPC can be used for therapeutic applications. First, there is the possibility that when allogeneic cells are implanted, a graft-versus-host immune reaction could occur, caused by a residual T-cell fraction derived from $CD34^+$ cells. Studies showed that

BM-derived $CD34^+$ cells can be differentiated into T cells both in vitro and in vivo [233]. CD34 is one of the most widely used surface makers used for EPC isolation. It is important to use CD34 combined with other surface markers to identify subsets of EPC that will be suitable for clinical use and to reduce the risk of subsequent T-cell differentiation. Second, to develop cells for clinical use, it is necessary to remove all animal substances, such as serum, from the culture environment, because they might be pathogenic or immunogenic. Conventional culture conditions use animal-derived components, especially sera (e.g., fetal bovine serum [FBS]). For clinical cell therapy applications, EPC culture conditions must be free of animal products to meet good manufacturing practice standards. Efforts were made to develop clinical-grade human embryonic stem cell lines and culture conditions [234]. Reinisch et al. [68] published results of clinically approved culture conditions to expand PB-EPC. Using pooled human platelet lysate to replace FBS in the culture environment, they reported the successful recovery of EPC (ECFC) from PB. The ECFC were characterized by robust proliferative potential (more than 30 population doublings), a normal karyotype, and vascular network-forming ability.

These summarized studies provide support for the presence of stem cells in PB and mechanisms by which they can be mobilized from BM to increase their numbers in blood. Although various attempts have been made to use PB-derived stem cells in humans and some encouraging results were obtained, standard clinical use of these techniques must await further validation and long-term toxicity evaluations.

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CHAPTER

21

From Adult Pancreatic Islets to Stem Cells: Regenerative Strategies for the Treatment of Diabetes and Its Complications

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INTRODUCTION

Pancreatic islet β cells synthesize and release the hormone insulin, which is fundamental for the control of glucose metabolism and glycemia. β cells can be lost; they can also be or become impaired as the result of different mechanisms. The lack or insufficiency of their function of insulin release leads to a group of diseases that share characteristic pathological features: an abnormal metabolism of carbohydrates and elevated levels of glucose in the blood and urine. For this reason, this group of diseases is collectively referred to as "diabetes mellitus," or more frequently, "diabetes." In this chapter, we review cell-based strategies that have been developed for the treatment of diabetes mellitus and its complications.

In a group of patients with diabetes, β cells are progressively lost as the result of an autoimmune process, and beta-cell function is largely lost. This form of diabetes mellitus is termed *type 1 diabetes* (T1D) [1]. To date, there is no definitive cure for this disease. In patients with T1D, life-long exogenous insulin therapy is required as a life-saving intervention. In the long term, most patients fail to achieve satisfactory metabolic control. Moreover, T1D patients remain exposed to the risk for developing both short- and long-term complications, some of which can be fatal [2]. A subset of patients with T1D cannot be managed effectively with exogenous insulin therapy and have dangerous swings in glycemia ("brittle" T1D). In these patients, diabetes can be ameliorated or corrected by transplantation of pancreas or pancreatic islets from cadaveric donors.

In another group of patients, β cells develop functional impairments and produce an insufficient amount of insulin in the context of insulin resistance. This form of diabetes, termed type 2 diabetes (T2D), largely results from diet and lifestyle choices; it is by far the most common form of diabetes: more than 90% of cases of diabetes in the United States are T2D. β cells can also be impaired if genetic conditions compromise their development or function, which can determine monogenic forms of diabetes or increase the susceptibility for other forms of diabetes, mainly T2D. In the most severe cases of T2D and of monogenic diabetes, beta-cell transplantation or regeneration strategies may be beneficial.

FROM ADULT PANCREATIC ISLETS TO STEM CELLS

Transplantation of pancreatic islets may be considered one of the oldest forms of cell transplantation. It is also one of the safest and least invasive transplant procedures. It can restore normoglycemia in patients with T1D [3], it abates to minimal levels the risk for hypoglycemia owing to insulin treatment [4], it leads to a marked improvement in patients' quality of life [5], it relieves symptoms of the disease for up to several years [6], and it slows or prevents

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disease progression [7]. Pancreas and pancreatic islet transplantations represent proofs of concept that transplantation of pancreatic islet cells can correct T1D in humans. These forms of transplantation present several limitations primarily connected to the low number of cadaveric organs available and the need for immunosuppression. Different forms of cell-based therapies aimed at beta-cell replacement for T1D are under investigation. β cells can be derived from stem or progenitor cells such as embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem/progenitor cells. Other regenerative strategies include reprogramming or the delivery of prodifferentiation signals. The design of differentiation methods is largely based on knowledge obtained from studies of embryonic and fetal islet development. Novel findings are emerging related to the existence of stem and progenitor cell niches in the adult human pancreas and to molecules that can stimulate their maturation into functional insulin-producing β cells. The involvement of these adult progenitor cells in normal tissue turnover and in regenerative phenomena in pancreas remains to be elucidated. This chapter will cover these fields. Moreover, we will discuss studies of the effect of mesenchymal stem cell (MSCs) transplantation in diabetes. MSCs can modulate immunity, preserve beta-cell function, and stimulate repair or regeneration of endogenous β cells. MSC had a good safety profile in initial clinical trials in patients with T1D; they are being tested also as a therapeutic option for complications of both T1D and T2D.

STRATEGIES FOR THE GENERATION OF β CELLS FOR REPLACEMENT THERAPY

The limited availability of donor pancreata and islets has prompted the search for alternative sources of β cells. Significant progress in developing strategies to direct cell differentiation has led to opportunities to generate β cells from different types of stem cells and provide high-yield supplies of β cells for replacement therapy. Diabetes mellitus is an attractive target for cell replacement strategies because a single cell type (insulin-producing β cells) is missing or insufficient; moreover, cells can be implanted using minimally invasive procedures and can also perform endocrine functions ectopically [8]. An unlimited supply of human β cells could extend currently used islet replacement therapy to millions of patients. Stem cell−derived β cells would also provide a unique platform for drug discovery and disease modeling for diabetes mellitus.

There are three major strategies for generating β cells in vitro [1]: directed differentiation [2], reprogramming (also referred to as transdifferentiation), and [3] transdetermination [1]. The directed differentiation strategy, which is the subject of intense studies, is based on the differentiation of stem cells into β cells through numerous intermediate stages, frequently in attempts aimed to recapitulate pancreatic development [2]. In the cell reprogramming strategy, terminally differentiated cells are converted into β cells without pluripotent or multipotent intermediate stages [3]. In the transdetermination strategy, adult multipotent stem cells or progenitors are induced to change the path of differentiation from a normal form closely related to β cells into β cells.

β CELLS FROM PLURIPOTENT STEM CELLS (EMBRYONIC STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS)

Pluripotent stem cells (PSCs), including both ESCs and iPSCs, possess the robust potential to self-renew and differentiate into multiple lineages. The abilities of these cells to proliferate (extensively in undifferentiated form and to differentiate into any cell type have led to the idea that large numbers of β cells could be generated in vitro by artificially directing the process. Nevertheless, directing PSC differentiation into glucose-responsive, insulin-secreting, mature β cells has proved to be more difficult than initially expected.

Currently used methods of generating β cells from PSCs in vitro are based on findings from studies of embryonic development. To generate β cells, undifferentiated PSCs are induced to differentiate through series of intermediate stages, including definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm, and pancreatic endocrine progenitors. This stepwise differentiation process recapitulating pancreas organogenesis can be directed in vitro by forced expression of defined transcription factors using DNA-based or non−DNA based strategies. The key transcription factors that direct undifferentiated progenitor cells toward fully mature, functional β cells have been identified over years of research in pancreatic development [9−15] (Table 21.1, Fig. 21.1). DNA-based reprogramming strategies use transgenic expression of key transcription factors to induce pancreatic lineage-specific gene expression and guide pancreatic endocrine cell differentiation. Transgenic expression of *pancreatic and duodenal homeobox 1* (*Pdx-1*), *neurogenic differentiation factor 1* (*NeuroD1*), *neurogenin 3* (*Ngn3*), and *musculoaponeurotic*

TABLE 21.1 Key Transcription Factors Involved in Pancreas and β-Cell Development

Pdx1	Pancreas and duodenum homeobox protein 1 (Pdx-1) is crucial for pancreas formation and β-cell differentiation.
Ngn3	Neurogenin 3 (Ngn3) is important for the development of the endocrine pancreas.
NeuroD1	Neurogenic differentiation factor 1 (NeuroD1) is required for pancreatic development and endocrine cell differentiation. It regulates expression of the insulin gene.
Nkx-6.1	Homeobox protein Nkx-6.1 is required for the development of β cells. It controls their expansion.
Nkx-2.2	Homeobox protein Nkx-2.2 is essential for the differentiation of pancreatic endocrine cells and is necessary for the maturation of β cells.
Pax-4	Paired box gene 4 (Pax-4) is involved in pancreatic islet development and in the formation of β and δ cells.
Pax-6	Paired box gene 6 (Pax-6) is necessary for β-cell differentiation and the normal expression of final differentiation markers such as insulin and glucose transporter 2.
MafA	The transcription factor musculoaponeurotic fibrosarcoma oncogene homolog A is important for the maintenance of β-cell function. In the pancreas, it is found predominantly in β cells.

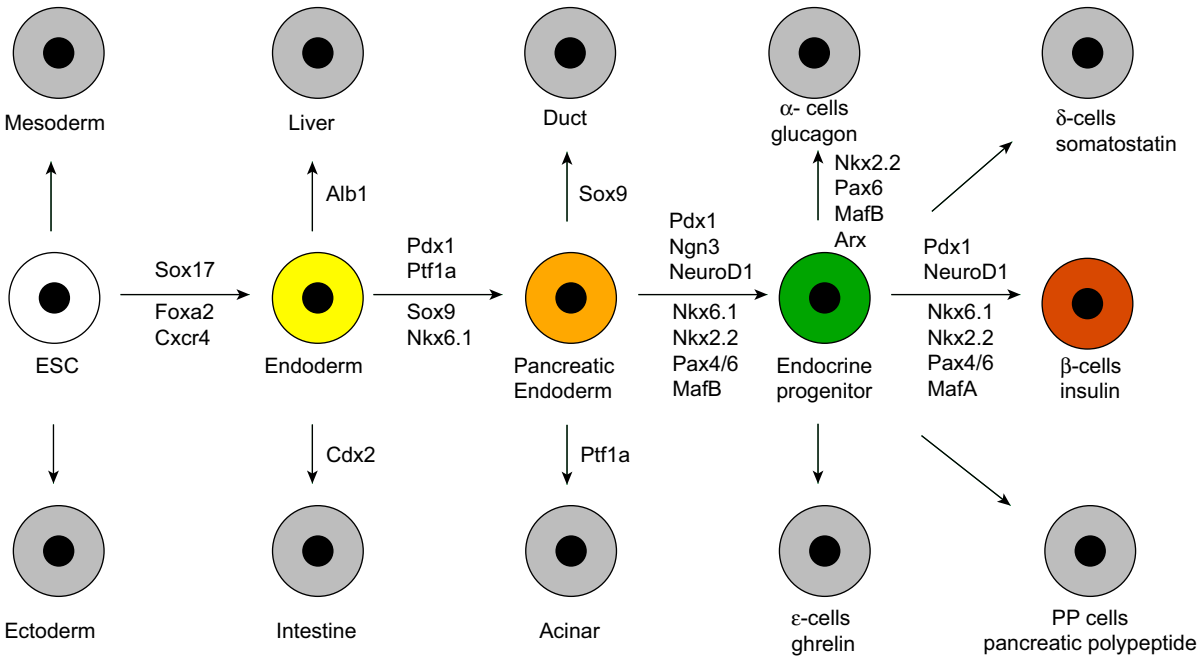

FIGURE 21.1 Pancreas and β-cell development. *ESC*, embryonic stem cell. The main transcription factors involved in fate determination are indicated along the arrows.

fibrosarcoma oncogene homolog A (MafA) has been used to differentiate ESCs into pancreatic endocrine cells, but potential risks for mutagenesis and oncogenesis associated with such genetic manipulation limit the clinical applicability of this method [16,17]. Therefore, non−DNA based strategies are preferred. The main strategies developed so far use signaling molecules (growth factors and small molecules) to activate or inhibit different signal transduction pathways leading to induction of key transcription factors specific for particular stages of pancreas development.

Early attempts to generate beta-like cells from ESCs were based on the spontaneous differentiation of ESCs [18,19] or on the selection and differentiation of cells positive for nestin, an intermediate filament protein expressed by neural progenitors [20−22]. Although pancreas and the central nervous system have different origins and functions, similar mechanisms control their embryonic development. On the basis of these similarities, it was hypothesized that strategies that induce the generation of neural cells from ESCs could be used to generate endocrine pancreatic cells [20]. In 2001, Lumelsky et al. reported for the first time the possibility of generating beta-like cells from mouse ESCs using a protocol based on the production of a highly enriched population of nestin-positive cells from embryoid bodies [20]. Two other groups repeated and improved these results and confirmed that ESCs could be

a source of new β cells [21,22]. However, insufficient characterization of the origin and phenotype of these cells, and later observation that insulin-positive cells did not produce insulin de novo but uptake it from the culture media, suggested that these progenitor cells could not be the reproducible source of bona fide β cells [23,24]. Lessons from these unsuccessful attempts to generate β cells emphasized the importance of understanding pancreas development, and especially the key signaling pathways that regulate stages of differentiation from PSCs to mature β cells in vivo. Therefore, efforts were made to control the differentiation process in vitro better by a stepwise manner that aims to recapitulate normal embryonic development in vivo.

In 2006, D'Amour et al. (Novocell, now ViaCyte) differentiated human ESCs into pancreatic hormone–expressing cells by directing them through definitive endoderm, gut-tube endoderm, pancreatic endoderm, and endocrine precursors stages [25] (Fig. 21.2). Cells derived with this protocol had strong expression of PDX1 and NK6 homeobox 1

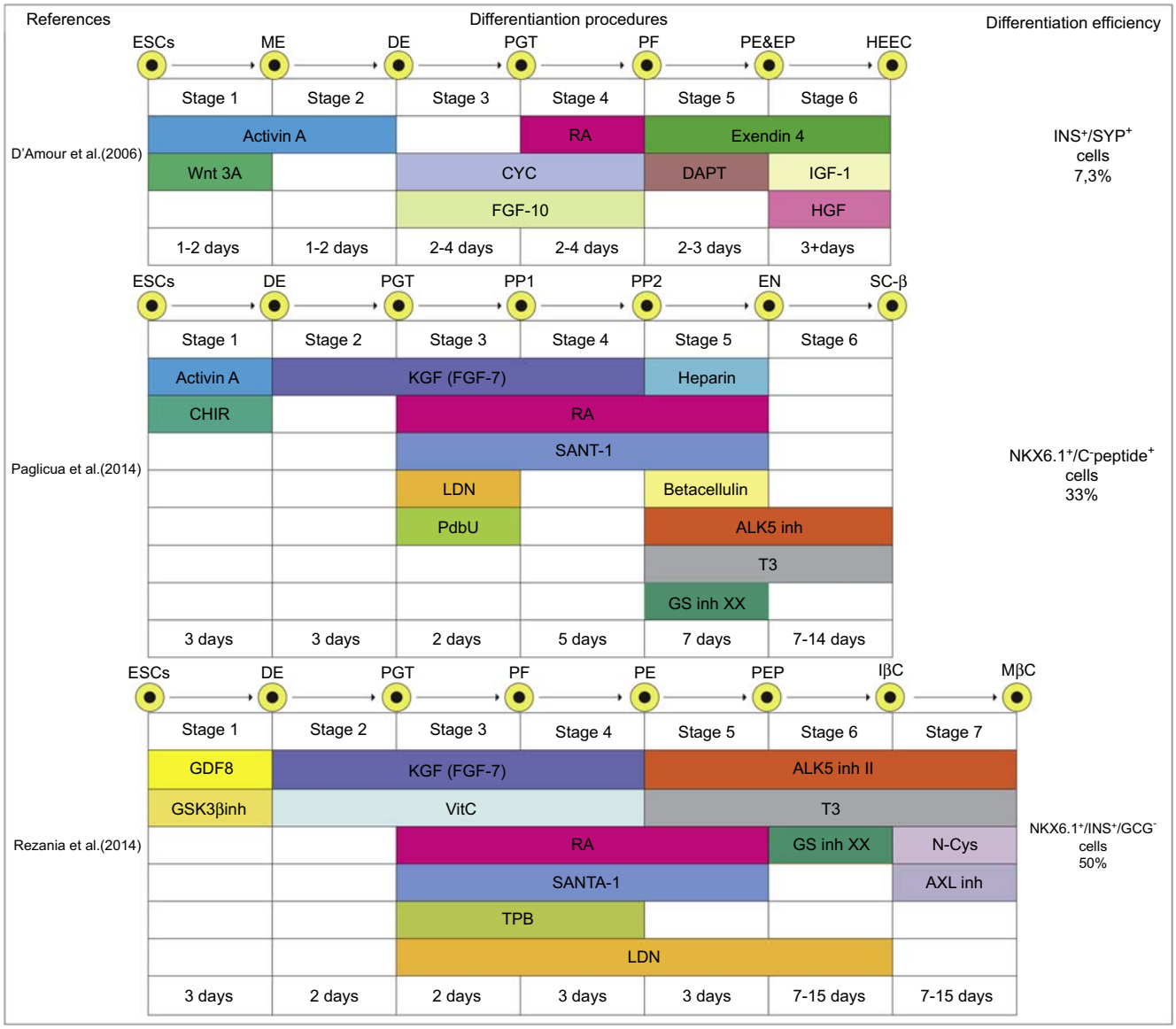

FIGURE 21.2 Scheme of main protocols for pancreatic β-cell differentiation from human embryonic stem cells. *A*; CYC, KAAD-cyclopamine; *AA*, activin; *AXL inh*, inhibitor of the tyrosine kinase receptor; *DAPT*, γ-secretase inhibitor; *DE*, definitive endoderm; *Ex4*, exendin-4; *FGF-10*, fibroblast growth factor-10; *FGF-7*, fibroblast growth factor-7; *GDF8*, growth differentiation factor-8; *GS inh XX*, gamma secretase inhibitor XX; *GSK3β inh*, glycogen synthase kinase 3 inhibitor; *HEECs*, hormone-expressing endocrine cells; *hESCs*, human embryonic stem cells; *IβCs*, immature β cells; *LDN*; bone morphogenetic protein receptor inhibitor; *ME*, meso endoderm; *MβCs*, maturing β cells; *N-Cys*, *N*-acetyl cysteine; *PdbU*, phorbol 12,13-dibutyrate, a protein kinase C activator; *PE&PP*, pancreatic endoderm and endocrine precursor; *PEP*, pancreatic endocrine precursor; *PF*, posterior foregut; *PGT*, primitive gut tube; *PP*, pancreatic progenitors; *RA*, all-trans retinoic acid; *SC-β*, stem cell–derived β cells; *T3*, triiodothyronine; *TPB*, PKC activator.

(NKX6.1), transcription factors known to be highly expressed in pancreatic progenitors and mature β cells but did not secrete insulin in response to glucose [25]. Further studies indicated that these progenitor cells could be differentiated into glucose-responsive, insulin-secreting cells in vivo 3 months after transplantation and prevent streptozocin-induced hyperglycemia in mice [26]. These results confirmed previous observations that human fetal (6- to 9-week) islet-like cell clusters can mature into glucose-responsive, insulin-secreting cells after implantation and correct hyperglycemia in diabetic mice [27,28]. Different modifications of this multistep protocol based on pancreatic developmental paradigms were developed further to differentiate both human ESCs (hESCs) and hiPSC into pancreatic progenitor cells capable of maturing and functioning in vivo [29–36].

Different agents (growth factors and signaling molecules) were used to activate or inhibit signaling pathways to induce the expression of set of transcription factors specific to particular developmental stages of β cells. The first fundamental step in differentiating PSCs into β cells is specifying the definitive endoderm. Wnt and transforming growth factor-β (TGF-β) signaling pathways have a crucial role in regulating definitive endoderm formation in vivo. Activin A (AA)/Nodal and Wnt3a are among the most commonly used factors to induce definitive endoderm in vitro [25,26,30–32,37]. It was indicated that a combination of growth differentiation factor 8 (a TGF-β family member) and glycogen synthase kinase 3 inhibitor (which activates Wnt signaling) is more efficient for inducing definitive endoderm formation than the combination of AA and Wnt3a [29]. The combination of AA with bone morphogenetic protein (BMP) 4 and fibroblast growth factor (FGF) 2 [34] or AA with an phosphoinositide 3-kinase inhibitor, either LY294002 [38] or wortmannin [39], induces definitive endoderm more efficiently than AA alone. Cells of a definitive endoderm can be identified by analyzing the expression of endodermal markers, including *SRY-related HMG-box 17 (SOX17), FOXA2* (also known as *HNF3ß*), *CXCR4, brachyury,* or *cerberus*. The next steps in differentiating PSCs into β cells are induction of the primitive gut tube and the posterior foregut. Activation of FGF and WNT signaling pathways, along with inhibition of *sonic hedgehog (SHH)*, are required during specification of the primitive gut tube in vivo. Inhibition of the BMP signaling pathway used to be included in differentiation protocols, but it was found to stimulate precocious differentiation and thus decrease the potential differentiation yield. Therefore, BMP inhibition was removed in certain attempts [40]. FGF7/KGF, FGF10, and 3-keto,*N*-aminoethyl aminocaproyl dihydrocinnamoyl (KADD)-cyclopamine are used upon removal of AA to pattern definitive endoderm into primitive gut tube in vitro [25,26,31]. At this stage, cells continue to express *FOXA2* and *SOX17*, markers for definitive endoderm, and start to express *HNF4α* and *HNF1ß,* markers of developing gut tube [41]. Activation of retinoic acid (RA) and FGF signaling pathways and inhibition of SHH (and, in certain cases, BMP) signaling pathways promote posterior foregut formation. To induce posterior foregut differentiation in vitro, the primitive gut tube cells are treated with RA, SANT (an SHH inhibitor), or KADD-cyclopamine (SHH inhibitor), Noggin or LDN193198 (BMP antagonists), and FGF10 [25,26,29,31,42]. Differentiated posterior foregut cells start to express *PDX1*, *HNF6*, *HLXB9*, *PROX1*, and *SOX9.* The next step in differentiating of PSCs into β cells is induction of pancreatic endoderm and endocrine progenitors. During normal in vivo pancreatic organogenesis, all pancreatic lineages including endocrine, exocrine, and ductal cells arise from *PDX1*-expressing progenitors. Induction of *NKX6.1* in these progenitors restricts their differentiation potential to become endocrine and ductal cells, while induction of *neurogenin 3* (*NGN3*) further restricts their differentiation potential to become endocrine cells [43]. Endocrine linage commitment is induced by the inhibition of BMP, TGF-β, and NOTCH signaling pathways. *PDX1*-expressing posterior foregut endoderm cells are recruited to pancreatic and endocrine lineages using different combinations of the γ-secretase inhibitor DAPT, compound E, or L-685.458 (NOTCH inhibitors), LDN193198 or Noggin (BMP inhibitors), and ALK5 inhibitor (TGF-β type I receptor kinase inhibitor). Other factors may have an important role at this step, because the use of forskolin (adenylyl cyclase activator), indolactam V or TATA-binding protein (protein kinase C activators), CYP26 A inhibitor, and vitamin A (precursor of retinoic acid) enhance differentiation process [29,31]. Endocrine progenitors at this step can be identified by analyzing the expression of *PDX1*, *NKX2.2*, *NKX6.1*, *NEUROD1*, *NGN3*, *paired box gene (PAX)4*, *PAX6*, and *MAFB.* The final step of pancreatic differentiation involves the maturation of endocrine progenitors into β cells. The key signaling pathways and factors that regulate β-cell maturation have not yet been fully discovered. This maturative step is frequently obtained in vivo after transplantation of cells at the progenitor stage. Proposed factors used to stimulate β-cell maturation in vitro include insulin growth factor (IGF)1, hepatocyte growth factor (HGF), glucagon-like peptide 1 or its agonist exendin 4, ALK5 inhibitor, LDN193189 (a BMP inhibitor), vitamin A, or CYP26 A inhibitor [25,29,44]. Mature β cells maintain the expression of *PDX-1*, *NKX6.1*, *NEUROD*, *PAX4*, and *PAX6* and increase robustly the expression of *MAFA,* and insulin (INS). Despite numerous modifications of the multistep protocol originally proposed by D'Amour and colleagues using different growth factors, small molecules, and cell lines, none were able to direct pancreatic progenitor cells in vitro toward fully mature, glucose-responsive β cells; instead, they became polyhormonal (insulin$^+$/glucagon$^+$/somatostatin$^+$) [29–36]. These polyhormonal cells displayed many characteristics of early fetal pancreatic cells, which suggests that they may reflect a normal embryonic

differentiation process [45,46]. Although it was indicated that in vitro generated ESC-derived polyhormonal cells differentiate into mature insulin-producing β cells after implantation, the signaling pathways regulating this process are unknown. Moreover, increasing evidence indicates that hESC-derived polyhormonal cells generated in vitro preferentially differentiate into functional glucagon-producing α cells rather than insulin-producing β cells after implantation [47].

Despite these ambiguities, the U.S. Food and Drugs Administration determined that it would be reasonably safe to test the transplantation of encapsulated ESC-derived pancreatic islet progenitors in humans. In 2014, the company ViaCyte started the first Phase 1/2 clinical trial with hESC-derived pancreatic progenitor cells to determine the safety, tolerability, and efficacy in patients with T1D. The purpose of that trial was to test the safety, tolerability, and efficacy of the VC-01 combination product in subjects with T1D with a 2-year (NCT02239354) and 3-year follow-up (NCT02939118) (https://clinicaltrials.gov). The VC-01 combination product consists of PEC-01 cells (pancreatic progenitors obtained from the hESC line) and the Encaptra drug delivery system (immune-protecting encapsulation device). Devices with pancreatic progenitor cells were transplanted subcutaneously in the area of the lower back of patients with T1D. The results of the clinical trial are awaited with great interest. In patients with T1D, the stem cell–derived β cells (SC-β cells) could be destroyed by the autoimmune process after implantation. Encapsulation devices could protect SC-β cells against a deleterious immune attack and could enable the function of transplanted β cells even in nonimmunosuppressed patients. Although the implantation of incompletely differentiated cells derived from human pluripotent stem cells (hPSCs) could create the risk for the formation of teratoma, the implantation of these cells in encapsulation devices provides an opportunity to remove them in case of complications. Numerous preclinical animal studies indicated that PSC-derived pancreatic progenitor cells mature into functionally insulin-producing β cells after implantation [30–33,35]. Ongoing clinical trials will determine whether pancreatic progenitor cells will differentiate efficiently into β cells after transplantation in patients with T1D and whether eventual maturation into undesired cell types will cause toxicity.

Significant progress has been made in extending the differentiation of PSCs to mature β cells. Two independent groups developed differentiation protocols that produce glucose-responsive, insulin-secreting cells in vitro [48,49]. Monohormonal populations of NKX6.1$^+$/C$^-$peptide$^+$ cells [49] or NKX.6.1$^+$/INS$^+$ cells [48] were generated with about 30% and approximately 50% efficiency, respectively (Fig. 21.2). In contrast, the previous protocols generated NKX.6.1$^+$/INS$^+$ cells with an overall efficiency of less than 1%.

The seven-stage protocol developed by Rezania and colleagues is based on the differentiation of PSCs (hESCs or iPSC lines) toward pancreatic progenitors PDX1$^+$/NKX6.1$^+$ (S1–S4), endocrine progenitors PDX1$^+$/NKX6.1$^+$/NEUROD1$^+$ (S5), immature β cells PDX1$^+$/NKX6.1$^+$/NEUROD1$^+$/INS$^+$ (S6), and finally, maturing β cells PDX1$^+$/NKX6.1$^+$/NEUROD1$^+$/INS$^+$/MAFA$^+$ (S7). The first difference between the new and previously published protocols was the generation of pancreatic progenitors maintaining a high coexpression of PDX1/NKX6.1 and a low expression of NGN3. Previously it was found that the suppression of NGN3 during early stages of differentiation prevented the formation of polyhormonal cells [50]. Therefore, the expression of NGN3 was inhibited at early stages of differentiation (S2–S3) by adding vitamin C. The addition of ALK5 inhibitor (a TGF-β receptor inhibitor), LDN193189, SANT, thyroid hormone (T3), and RA at stage 5 (S5) to the air–liquid interface cultures of PDX1$^+$/NKX6.1$^+$ cells led to their differentiation into endocrine precursors maintaining robust coexpression of PDX1/NKX6.1 and NGN3, NKX2.2, or NEUROD1 markers. Up to 50% of cells generated with this protocol coexpressed NKX6.1/NEUROD1. Previously published protocols did not generate cells expressing endocrine precursor markers NEUROD1 or NKX2.2 at later stages in vitro. Further exposure of NKX6.1$^+$/NEUROD1$^+$ cells at S6 to gamma secretase inhibitor XX (GSiXX) (a NOTCH inhibitor) induced the expression of genes involved in β-cell maturation and inhibited expression of *PTF1A*, a marker of the pancreatic exocrine lineage (during the late stages of maturation). Combining GSIXX with T3 resulted in the generation of cell populations containing about 50% of NKX6.1$^+$/insulin$^+$/glucagon$^-$ cells. However, these cells did not secrete insulin in response to glucose in vitro. Applying an inhibitor of the tyrosine kinase receptor AXL at S7 induced expression of *MAFA*, a marker of mature β cells, at a level similar to that of human islets. Approximately 50% of cells obtained at S7 were insulin positive. This protocol enables the generation of PDX1$^+$/NKX6.1$^+$/NEUROD1$^+$/INS$^+$/MAFA$^+$ cells with an insulin content, glucose-stimulated insulin secretion, and a structure similar to that of human islet cells. However, the insulin and calcium response to glucose were delayed compared with human islet β cells. Despite these differences from human islet β cells, the S7 cells were capable of rapidly reverse diabetes in vivo [48].

Pagliuca et al. applied a scalable suspension-based culture system able to generate hundreds of millions of glucose-responsive human β cells. This six-staged protocol is based on the differentiation of PSCs (hESC and hiPSC lines) into pancreatic progenitors (S1–S4), endocrine progenitors (S5), and SC-β cells (S6). To generate highs numbers of pancreatic progenitors PDX1$^+$/NKX6.1$^+$ (>55% of cells), the group extended the time in culture with

KGF (FGF-7), SANT1, and RA. Further exposure of $PDX1^+/NKX6.1^+$ cells to ALK5 inhibitor, GS inhibitor XX1, T3, SANT1, and betacellulin (an epidermal growth factor [EGF] receptor ligand) at S5 followed by T3 and ALK5 inhibitor at S6 led to the generation of an average of 33% $NKX6\text{-}1^+$/C-$peptide^+$ SC-β cells. These SC-β cells fluxed Ca^{2+} and secreted insulin in response to glucose and displayed a gene expression pattern and ultrastructure similar to primary human β cell. Furthermore, SC-β cells were able to reverse hyperglycemia in diabetic mice [49].

Significant progress in the generation of β cells in vitro was made by Rezania and colleagues and Pagliuca and colleagues, but in vitro–generated β cells were not fully equivalent to mature β cells and the long-term function of these cells after implantation remains unknown. Therefore, further lessons have to be learned from pancreas development during prenatal and postnatal life generate fully functional β cells.

Stepwise pancreatic differentiation protocols were applied successfully to hiPSCs from nondiabetic individuals [39,48,49,51,52], patients with T1D [53,54] and patients with genetic forms of diabetes (maturity-onset diabetes of the young type 2) [55]. These studies indicate that a genetic predisposition for diabetes is not an obstacle in generating β cells in vitro. iPSCs derived β cells from patients with diabetes provide a platform for analyzing specific genotypes on β-cell function and enable gene correction strategies [55]. The differentiation efficiency of iPSCs for β cells differs significantly between patients [53,54], but this variability is also observed between different hESC lines [31,37,39,51]. Differentiation protocols with better consistency in yields may be developed in the future.

β CELLS FROM ADULT STEM/PROGENITOR CELLS OF THE BILIARY TREE AND PANCREAS

Studies in animal models indicate that the repair of mild and moderate injuries in the pancreas is supported mainly by the replication of mature parenchymal cells. Nevertheless, pervasive and chronic injuries could stimulate a contribution from more undifferentiated stem or progenitor cells. If such cells exist, they could be harnessed to treat patients with diabetes. The existence, phenotype, and anatomical location of stem and progenitor cells in the adult human pancreas are at the center of an active discussion.

Precursor cells for the pancreas, liver, and biliary tree exist at early stages of human development, in the endoderm forming the *foregut* [56,57]. From the foregut, a structure emerges, termed the *hepatic diverticulum* [58]. The cranial portion of this structure forms the rudiments of liver and common hepatic duct, developing into cells that will mature into hepatocytes and cholangiocytes. The caudal portion of the hepatic diverticulum, connected to the foregut, gives rise to the *extrahepatic biliary tree* and to the *ventral pancreatic bud*; this last bud will become the ventral portion of the pancreas. The caudal portion of the diverticulum harbors biliopancreatic stem and progenitors cells coexpressing SOX17 and PDX1. Subsequent segregation of pancreatic and biliary precursors depends on these two transcription factors [59]. In an opposite region of the foregut, the *dorsal pancreatic bud* emerges. Dorsal and ventral pancreatic primordia derive from PDX1-positive pancreatic stem and progenitor cells [60]. These progenitors branch into primitive epithelial tubules [60]. Exocrine acini derive from "tip progenitors" located at the ends of primitive tubules. Development of endocrine cells begins in the central duct area, where "trunk progenitors" transiently express NGN3 and commit to an islet fate [60]. On the basis of the progenitor segregation, multiple stem cell niches seem to persist postnatally in specific niches. Stem and progenitor cells have been observed within the extrahepatic biliary tree and in association with intrapancreatic ductal structures (Fig. 21.3A). The most primitive of the stem and progenitor cells, termed biliary tree stem cells (BTSCs) [61], are found in peribiliary glands (PBGs) within extrahepatic bile ducts, and primarily at branching points and in the region of the hepatopancreatic duct [62]. PBGs are crypt-like invaginations of the extrahepatic bile duct epithelium [56,61,63,64]. These niches harbor cells with markers of multipotency for pancreatic and liver maturation (SOX17, PDX1, and SOX9), and with surface markers related to endodermal stem and progenitor cells (epithelial cell adhesion molecule [EpCAM] and leucine-rich repeat-containing G-protein coupled receptor 5) (Fig. 21.3B). BTSC cells can be observed in (and may be isolated from) the fetal and adult biliary tree. They are capable of self-replication and multipotent differentiation toward pancreatic cells (including functional islet endocrine cells) and mature liver cells (hepatocytes and cholangiocytes) both in vitro and in vivo [56,62,64,65]. In the pancreas proper, more mature cells are observed: pancreatic ducts and pancreatic duct glands (PDGs) harbor committed pancreatic progenitors [66] (Fig. 21.3C). These cells could contribute to pancreatic organogenesis throughout life.

Niches containing cells related to BTSCs extend into liver and near the pancreas [64,65]. The largest pancreatic ducts display PDGs. Detailed anatomical studies in humans have revealed a proximal-to-distal axis for the BTSC niche organization going from proximal (the hepatopancreatic ampulla), the location of the most primitive stem cells, to distal (the liver or the pancreas), the location of mature cells [62,64,65]. This axis recapitulates the

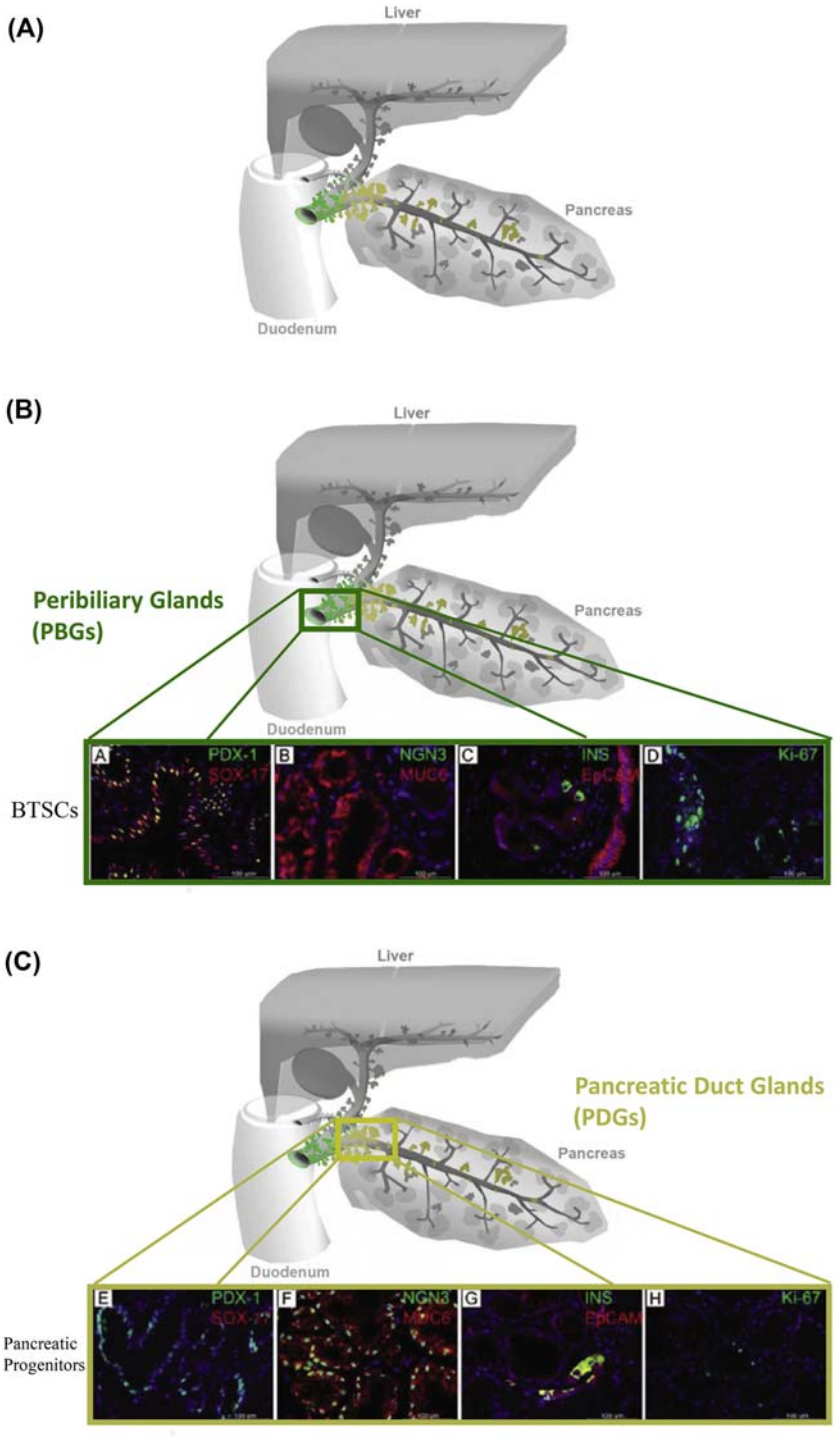

FIGURE 21.3 (A) Schematic representation of the pancreas, liver, duodenum, along with the biliary tree and network of pancreatic ducts. Peribiliary glands (PBGs) are marked in *green* and pancreatic duct glands are marked in *yellow-gold*. (B) PBGs exist along the hepatopancreatic duct harbor biliary tree stem cells (BTSCs), as well as more mature derivatives. PBGs display (A) cells with a range of pancreatic and duodenal homeobox 1—positive ($PDX1^+$) and SRY-related HMG-box 17—positive ($SOX17^+$) expression, (B) subsets of cells that are mucin 6, oligomeric mucus/gel-forming positive ($MUC6^+$) and $MUC6^-$, but negative for the islet progenitor marker NGN3, (C) scattered Insulin + cells embedded in the epithelial cell adhesion molecule-positive epithelium, and (D) several Ki-67-positive proliferating cells. (C) Pancreatic duct gland (PDGs), distributed along the largest pancreatic ducts, harbor pancreatic progenitors, including subsets of putative progenitors committed to islet endocrine phenotypes. PDG display (E) a large number of $PDX1^+$ cells, with low or no expression of SOX17, (F) subsets of $NGN3^+$ $MUC6^+$ cells, putative islet endocrine progenitors, (G) clusters of insulin-positive cells, (H) a limited number of Ki-67-positive proliferating cells.

organogenesis of these endodermal organs; it is based on their common embryological origin [56] and is in accordance with the presence of a stem cell compartment shared and in common for the pancreas and the liver [67].

Human BTSCs are endowed with the potential to mature into functional insulin-producing cells that ameliorate diabetes in rodents. For this reason, they are a candidate source of cells for regenerative strategies in T1D [65,68]. Beside cell therapy, the modulation of BTSC differentiation toward β cells via growth factors or other signals represents a possible therapeutic target [69].

Postnatally, islet cell proliferation is sustained after birth, but turnover becomes slow in adulthood. However, compensatory growth can occur in response to increased demand in both the endocrine and exocrine compartments [70,71]. The question remains related to the nature of the cells responsible for such regenerative phenomena. Few reports claimed that multipotent stem cells exist within the pancreas [72,73], but such cells are rare in the postnatal pancreas [74]. Cells with pancreatic stem cell traits seem to persist postnatally in the human biliary tree, especially in the region of the hepatopancreatic duct and of the ampulla of Vater, whereas more committed progenitors appear to reside in the pancreas proper [62]. Before the observations on the biliary tree niche of multipotent stem cells for the pancreas, investigators focused on whether mature pancreatic cells could be the source of regenerative phenomena. Pancreatic cells were hypothesized to be endowed with a flexible differentiation potential to respond to injuries. A number of studies suggested that in adults, new pancreatic cells arise mainly from preexisting cells of the same lineage. Lineage tracing studies in rodents showed that acinar cells can develop into to acinar cells but not islets [75]. Similarly, newly formed islet β cells were found to derive mainly from the replication of preexisting islet β-cells [76,77]. These findings do not preclude a contribution from multiple populations of stem or progenitor cells for the de novo formation of exocrine and endocrine cells, a contribution frequently observed after extensive and chronic damage [62,72,73,78,79]. β-cell impairment or loss, peripheral insulin resistance, and hyperglycemia may stimulate regeneration from endogenous progenitors [80].

The epithelium of most pancreatic ducts is composed of Sox9+ and carbonic anhydrase II^{+} cells [67]. Adult ductal cells were reported to be able to differentiate into pancreatic acinar and endocrine cells [78,81]. However, the contribution of this population to islet endocrine cell turnover is still a matter of debate [67,78,81]. Intriguingly, in response to pathological conditions that include pancreatitis and autoimmune diabetes, structures with heterogeneous ductal phenotypes expand in the pancreas, with a histological presentation that resembles liver ductular reactions [82,83]. PDGs of the adult human pancreas were found to harbor pancreatic endocrine progenitors [62]. PDGs occur in association with the main pancreatic duct and its immediate ramifications, and are abundant in proximity to the hepatopancreatic ampulla [62,84]. The anatomical distribution of PDGs along the pancreatic ducts of larger caliber has similarities to the distribution of PBGs along the biliary tree. PDGs harbor NGN3-positive cells, putative progenitors of islet cells [62]. PDGs undergo hyperplasia in murine models of chronic pancreatitis, displaying traits of mucinous intestinal metaplasia, and pancreatic intraepithelial neoplasia [84]. Hence, cells in adult human PDGs and along the larger pancreatic ducts have a phenotype consistent with that of pancreatic progenitors. Considered altogether, these observations suggest that the adult pancreas contains niches of progenitors that could mediate regenerative responses.

Studies have identified growth factors that can stimulate the maturation of insulin-producing β cells from adult pancreatic nonendocrine cells, including putative progenitor cells. A study was conducted in mice after the induction of diabetes with the chemicals alloxan or streptozotocin. EGF and ciliary neurotrophic factor were administered intraperitoneally. This resulted in diabetes reversal in 64% of the animals rendered diabetic with alloxan, and in 33% of animals rendered diabetic with streptozotocin [85]. A different study was conducted on cultured human nonendocrine cells, obtained as a by-product of islet isolation. This study analyzed the effect of BMP-7. BMP-7 is a growth factor in clinical use, with the dual ability to inhibit TGF-β and activate BMP signaling. Exposure of human nonendocrine pancreatic cells to BMP-7 led to the generation of glucose-responsive, insulin-releasing cell clusters that expressed insulin within the published range for research islets [86,87]. Preliminary lineage-tracing results suggested that cells that responded to BMP-7 were not adult acinar or ductal, but rather were pancreatic progenitor cells. Further anatomical and molecular characterization of these cells may shed additional light on their potential to regenerate the endocrine pancreas. Linking this field of research to that of ESC differentiation was the finding by Russ et al. [40] that BMP inhibition (commonly used in earlier methods to differentiate ESCs into β cells to promote efficient pancreatic progenitor cell generation) results in dysfunctional or polyhormonal β-like cells. Removal of these inhibitors corrected this problem, which further suggests that BMP signaling is important for the functional differentiation of β cells.

MESENCHYMAL STEM CELLS TO MODULATE IMMUNITY AND PROMOTE TISSUE REPAIR IN DIABETES

MSCs have created great interest. They possess important characteristics that could be exploited in cell-based strategies for T1D and for certain complications of T2D. MSCs are potent immunomodulators. They exert suppressive functions on immune effector cells and orchestrate the action of other regulatory cells. Moreover, they promote the repair and regeneration of endogenous tissues in recipients (Fig. 21.4). Furthermore, they have shown a good safety profile in clinical trials, including a limited risk of tumor formation. MSC transplantation has been tested extensively and has been shown to be beneficial in animal models of autoimmunity [2,88,89].

MSCs are endowed with the ability to modulate immune responses. Several mechanisms of action have been described. MSCs interact with immune cells such as dendritic cells (DC), natural killer cells, β cells, and T cells [90]. MSCs can inhibit DC differentiation and maturation, suppress the proliferation of $CD4^+$ and $CD8^+$ T cells, and impair the cytotoxic activity of cytotoxic lymphocytes [2]. MSCs can also induce and expand T-regulatory (T-reg) cells and can balance T-helper (Th) subsets [91]. Studies conducted in the Non Obese Diabetic mouse model (a model of autoimmune T1D) showed that MSCs can induce FoxP3+ T-reg cells that secrete the antiinflammatory cytokine interleukin (IL)-10 [92]. Moreover, MSCs could expand antigen-specific T-reg cells in vivo and stimulate long-lasting tolerance [93]. MSCs are also able to shift the cytokine profile from proinflammatory to antiinflammatory in the murine pancreatic microenvironment, polarizing the response from Th1 to Th2 in T1D animal models [2]. The ability of MSCs to modulate immunity should not be considered solely immunosuppressive; it depends closely on the microenvironment: MSCs acquire proinflammatory activity when homed into a low-inflamed microenvironment [91,93].

Dr. Arnold Caplan, who coined the nomenclature "mesenchymal stem cells" and the MSC acronym in the late 1980s, later preferred to call these cells "medicinal signaling cells." This is because MSCs have a prominent secretive activity. Great effort has been made to understand the effects of releasing these bioactive molecules. Interestingly, MSCs can home into inflamed tissues as a result of the interaction of receptors with ligands, which indicates inflamed environments, such as CXCR4 with its ligands chemokine (C-C motif) ligand-12, and stromal cell−derived factor-1, and very late antigen-4 with vascular cell adhesion molecule-1 [93]. MSCs secrete a number of bioactive molecules at their homing site, including IL-6, IL-8, TGF-β, nitric oxide, indoleamine 2,3-dioxygenase, tissue inhibitor of metalloproteinases-2, vascular endothelial growth factor, HGF, granulocyte macrophage−colony-stimulating factor, basic fibroblast growth factor, and insulin-like growth factor-binding protein (IGFBP)3, 4, and 7 [93,94]. These molecules can promote tissue repair and act as chemoattractants, recruiting macrophages and endothelial cells at the site of injury or inflammation. In addition, MSCs can signal through cell contact and via microvesicles, mechanisms that participate in immunoregulation [95]. Studies in rodent models of T1D seem to validate the hypothesis that MSCs boost endogenous tissue regeneration, because mice transplanted with human MSCs displayed higher serum insulin than do the control group, but in absence of human insulin [88]. Several clinical trials have tested MSC transplantation in patients with T1D and T2D [2] [65].

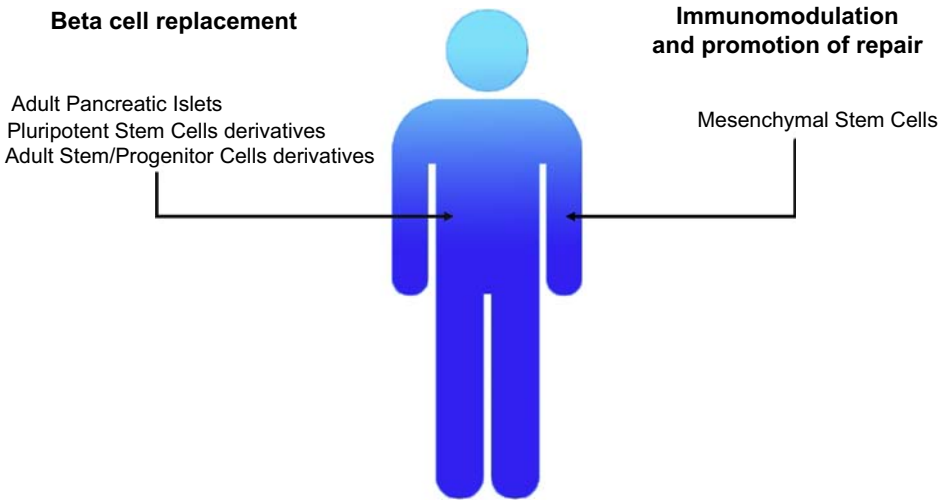

FIGURE 21.4 Visual summary of the main strategies for β-cell replacement, along with cell-based strategies for immunomodulation and to promote tissue repair.

As mentioned, T1D is a chronic autoimmune disease in which β cells are progressively lost, leading to severe complications such as hyperglycemia and ketoacidosis. Major hurdles for effective treatment of T1D are to halt immune destruction of β cells, preserve β-cell function and mass, and regenerate or replace β cells [1]. Because of their properties, MSCs could achieve these goals. Clinical trials have been performed to test the effect of MSC transplantation at different stages of T1D (Table 21.1). An open-label pilot trial [96] conducted in Sweden enrolled patients with T1D with recent onset of diabetes. Twenty patients were randomized to the group receiving transplantation of autologous bone marrow-derived MSCs or to the control group that received only insulin therapy. The treatment was found to be safe. Remarkably, most patients treated with MSC transplantation showed preservation of stimulated C-peptide secretion (a measurement of the function of insulin release from residual β cells) at 1 year after MSC infusion. The control patients showed a decline in C-peptide levels during the 1-year follow-up. Patients in both the treated and control groups continued to require insulin therapy and there were no statistically significant differences in insulin requirements and glycated hemoglobin (HbA1c) levels between the two groups. Another trial was conducted in China [97] and enrolled 42 patients with T1D who had diabetes for an average of 8.12 years. Compared with newly diagnosed patients, these patients had a severely lower level of residual β-cell function. The patients were randomized to a group receiving cell transplantation or one receiving standard diabetes care. The treated patients received cotransplantation of allogeneic umbilical cord−derived MSCs (UC-MSCs) and autologous bone marrow mononuclear cells (BM-MNCs). Given the design of the trial, the questions remain unanswered, because the individual actions of UC-MSCs and BM-MNCs could not be determined. This phase I/II trial aimed to test the safety and efficacy of this form of cell transplantation. Efficacy was assessed by measuring stimulated C-peptide, HbA1c levels, and insulin requirements. The therapy was found to be safe and resulted in a moderate improvement in β-cell function and metabolic parameters. After 1 year, stimulated C-peptide release during an oral glucose tolerance test was increased by 105.7% in recipients of cell transplant; in contrast, it declined in control patients. Moreover, HbA1c decreased by 12.6% in the treated group whereas it increased by 1.2% in the control group. Fasting blood glucose levels decreased significantly in transplant recipients, whereas it remained substantially unchanged in the control group. A change in the pattern of cytokine expression suggested that this form of cell therapy exerts immunomodulatory effects. Such improvements were achieved in patients with established T1D with fasting C-peptide less than 0.1 pmol/mL at entry. The reported improvement suggests some effect of the therapy on the mass of β cells, perhaps through the expansion of the residual β cells or differentiation of the transplanted cells into new β cells (although there is limited and controversial evidence for the differentiation of MSCs into pancreatic islet cells in vivo). The safety of MSC transplantation was corroborated in these clinical trials in patients with T1D [96,97]. The positive outcomes encourage larger, long-term studies of MSC transplantation in newly diagnosed and long-standing patients with T1D.

Ongoing clinical trials are also testing the safety and efficacy of MSC transplantation in patients with T2D [65,98]. MSCs could treat common complications of diabetes such as ulcers, limb ischemia, and nephropathy, and improve metabolic control in T2D [98−101]. Treatment with MSCs was reported to be safe and effective in facilitating wound healing in diabetic patients with foot ulcers [102], and the cells can exert an immunomodulatory effect by inducing T-reg cells in patients with T2D [103]. Allogeneic placenta-derived MSCs were transplanted in 10 patients with T2D and the infusion was reported to be safe. The patients experienced a reduction in the daily requirement for insulin; they had better control of blood glucose fluctuations and experienced improvements in quality of life [104]. Allogeneic adipose tissue-derived cells (not thoroughly characterized as MSCs) were also used in combination with bone marrow−derived cells in patients with insulin-dependent diabetes mellitus [105,106]. In that study, pancreatic endocrine differentiation was stimulated in vitro and induced adipose tissue-derived cells were co-infused in patients with culture-expanded bone marrow cells. No adverse side effects related to the infusion were observed, and transplantation was reported to result in reduced insulin requirements in the treated patients. Unfortunately, that particular study had caveats: the nature of the transplanted cells was not fully defined, the cell dose was not reported, and the mechanisms remained obscure, which raises some concerns regarding interpretation of the outcomes. Future studies are expected to provide a rigorous assessment of the efficacy of MSC transplantation in T1D, T2D, and related complications. Moreover, future efforts aim to elucidate the biological mechanisms of the effects of MSC transplantation in diabetic patients.

CONCLUSION

Thirty years of studies in islet transplantation paved the way to the transplantation of exogenously generated β cells as effective treatment for diabetes, chiefly in T1D. Some avenues of research are translating into clinical trials. The derivation of pancreatic progenitors from hESC proved successful in preclinical studies, and the safety of

encapsulated ESC derivatives is being tested in human recipients. Approaches are also emerging to stimulating the maturation of adult human pancreatic progenitors into functional endocrine cell types. Strategies that use cytokines or growth factors may soon reach the clinical testing phase. Regenerative approaches may not work efficiently in patients with T1D unless steps are taken to counteract autoimmunity. MSCs can act as potent immunomodulators and release trophic mediators. They are showing promising results in initial clinical studies in patients with T1D. MSCs can also stimulate the regeneration of endogenous cells in recipients, and findings in clinical trials with patients with T1D and patients with complications of T2D provide hints in that direction. There is no doubt that regenerative advanced therapies for diabetes are taking on momentum.

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CHAPTER

22

Stem Cells for Diseases of the Retina

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INTRODUCTION

Retinal disorders represent a leading cause of permanent vision loss worldwide. Unfortunately, no approved treatments exist for this group of disorders, resulting in a huge unmet medical need. Diseases such as atrophic or dry age-related macular degeneration (AMD), Stargardt macular dystrophy (STGD), and retinitis pigmentosa (RP) feature an initial period of cellular dysfunction followed by frank cell loss that is the ultimate cause of permanent blindness. Transplanting replacement cells for these conditions represents an attractive therapeutic paradigm because it may be agnostic to the underlying pathophysiology. For example, although AMD and STGD feature disparate underlying disease processes, both disorders ultimately result in the loss of the retinal pigment epithelium (RPE), which represents a common target for cellular transplantation. Human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) are promising sources from which to create fresh RPE cells that could be used to replenish compromised areas of the retina or even replace atrophic areas that are devoid of RPE. Other disorders that primarily feature photoreceptor degeneration such as RP may be treatable with stem cell–derived photoreceptor transplants. Because over 120 genetic variants of RP exist, this treatment approach might obviate the need to create custom treatments for each subtype.

Various stem cell types are being tested as sources of neurotrophic factors to stop or delay ongoing cellular loss. Human umbilical tissue-derived stem cells (hUTCs) as well as fetally sourced cells such as retinal progenitor cells (RPCs) and neural stem cells have shown promise for slowly progressive disorders such as dry AMD and RP. Adult stem cells such as bone marrow–derived hematopoietic stem cells have been employed in an autologous fashion for these degenerative conditions as well as for retinal vascular diseases. Any treatment strategy must be carefully scrutinized because there have been several cases of questionable stem cell therapies administered with minimal to no scientific validation or oversight [1].

In addition to studying stem cell–derived cellular products as potential therapeutic agents, scientists have become increasingly adept at differentiating stem cells into various retinal cell types in vitro. The capacity to recapitulate diseased retinal cells in culture (so called "disease-in-a-dish" modeling) has shown promise in characterizing particular patients' retinal disorders. Pluripotent stem cells can even be used to create three-dimensional optic vesicle-like structures that contain multiple retinal cell types in a laminar arrangement. This technology offers an unprecedented window into fetal retinal development at the cellular, developmental, and synaptic levels and may even serve as a source of cells for therapeutic use. The use of these in vitro differentiation methods can introduce artifacts, however, and any conclusions must be tempered with caution.

Stem cell–based translational research efforts have the potential to revolutionize the treatment and understanding of retinal disease, especially disorders with no treatments currently available. Furthermore, because the retina is embryologically derived from the central nervous system, it is likely this work will inform and instruct the use of cellular therapy in the brain. Compared with other central nervous system structures, the retina is more accessible, directly visualized, amenable to local immunosuppression, and requires fewer transplanted cells. These distinct advantages uniquely position retinal researchers and translational scientists to pave the way for cell-based treatments addressing a broad range of neurodegenerative diseases.

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The Retina

The human eye is a complex organ composed of multiple elements meant to capture light from the environment and transmit to the brain in the form of neuronal stimuli. The cornea and lens focus incoming light onto the retina, which converts light into electrical responses, a process known as phototransduction. This information exits the retina to the brain in the form of electrical signals via the optic nerve, which is composed of retinal ganglion cells whose nuclei reside in the retina (Fig. 22.1).

The retina is a multilayered transparent structure that is embryologically derived from the inner and outer layers of the optic cup. The key function of the retina is to perform phototransduction via the photoreceptor cells, but the other resident cell types, both neuronal and nonneuronal, have important roles such as signal processing, transmission of neuronal stimuli to the central nervous system, and regulation of metabolic function (Fig. 22.2A). The macula is the central portion of the retina bounded by the vascular arcades and the optic disc. A central depression within the macula is known as the fovea and is responsible for high-acuity color vision mediated by cone photoreceptors. The retinal periphery contains mostly rod photoreceptors and provides peripheral vision especially in low-light conditions.

The outermost layer of the retina is the RPE. The RPE consists of polarized, pigmented epithelial cells that reside between the highly vascular choroid and the photoreceptor layer. It provides several essential supportive functions to the photoreceptors including maintenance of the blood−retina barrier, phagocytosis of photoreceptor outer segments, absorbing scattered light, secretion of growth factors and cytokines, and metabolism of vitamin A derivatives [2]. The RPE monolayer exhibits apicobasal polarity with the apical processes in contact with the photoreceptor outer segments and the basal side abutting the choriocapillaris, a rich vascular plexus. The basement membrane of the RPE, which lies between the choriocapillaris and the RPE basal surface, serves as a porous matrix for adequate diffusion of nutrients. In the elderly, it undergoes deleterious changes such as atrophy, thickening, lipid accumulation, and diminished diffusional capacity, which may have a role in diseases such as AMD [3].

The photoreceptor layer contains rods and cones, which are highly specialized neuroepithelial cells that transduce light into electrical potentials. Rods are sensitive to dim light and make up most of the photoreceptors in the peripheral retina. Cones provide high-acuity color vision in bright light conditions and are highly concentrated in the fovea. Hence the retina mediates both central fine color vision and peripheral low-light sensitivity. The rods and cones perform phototransduction via outer segments that abut the apical processes of the RPE. Changes in membrane potential triggered by light stimuli are transmitted to synapses with bipolar cells, which in turn relay these signals

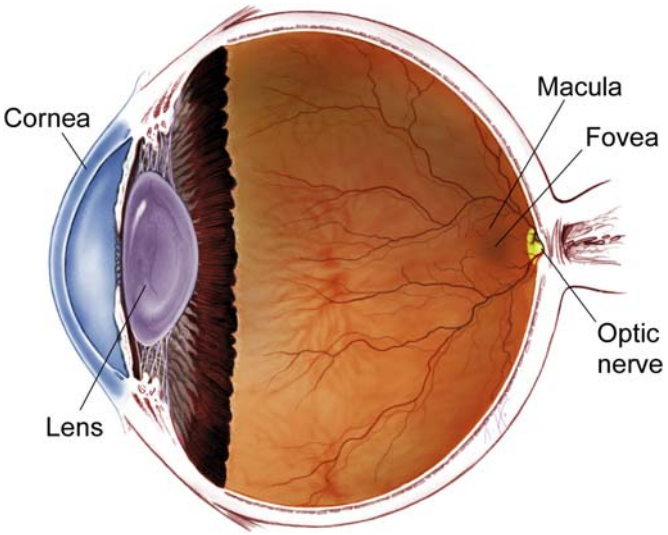

FIGURE 22.1 The eye contains multiple elements required for sight. The cornea and lens refract incoming light rays onto the retina. The retina is an extension of the central nervous system evolved to sense light. The retina can be grossly divided into a central portion and peripheral portion. The central retina, the macula, is bounded by the large vascular arcades emanating from the temporal aspect of the optic disc and is responsible for cone-mediated, high-acuity central vision. The peripheral retina is responsible for rod-mediated visual functions such as night vision, tracking, and peripheral vision. The visual cycle occurs in the photoreceptors and retinal pigment epithelium, initial signal processing occurs in the inner layers of the retina, and the optic nerve, composed of retinal ganglion cell axons, transmits the neural signal to the brain. *Illustration by Timothy Hengst.*

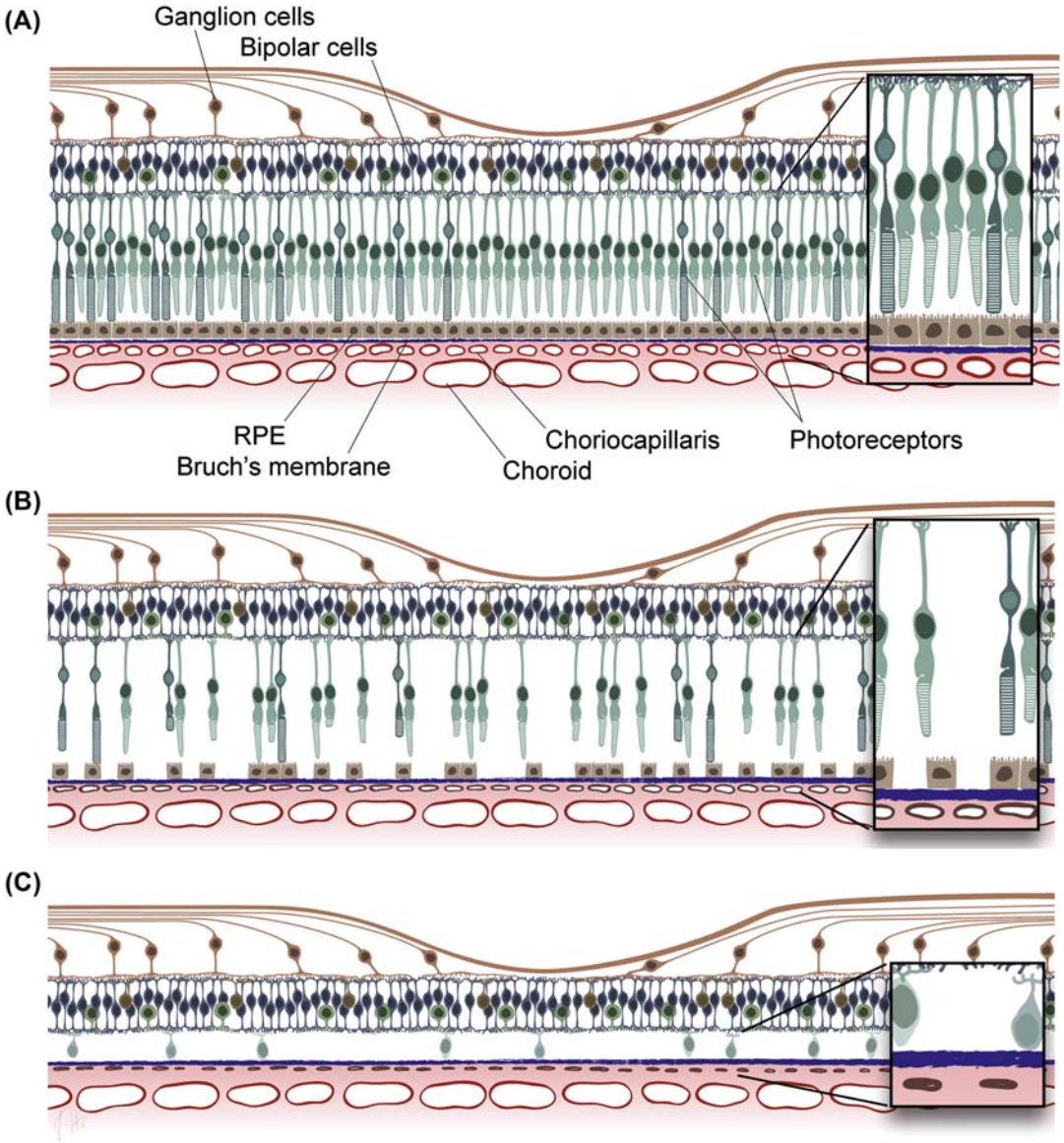

FIGURE 22.2 Schematic cross-sectional view of the normal and diseased macula. (A) The neurosensory retina consists of many cell types, including rod and cone photoreceptors, bipolar cells, and retinal ganglion cells. Note that the photoreceptor outer segments are in contact with the apical processes of the retinal pigment epithelium (RPE) monolayer (inset). The polarized, confluent monolayer of RPE cells rests upon the Bruch membrane and the innermost portion of the choroidal vasculature, the choriocapillaris. (B) The diseased retina in atrophic age-related macular degeneration (AMD), for example, may exhibit a diminution of RPE and photoreceptors along with a thickened Bruch membrane and drusen (metabolic and inflammatory debris; not illustrated). The underlying choriocapillaris can be thin or atrophic. Note that the remaining photoreceptors are compromised with shortened outer segments (inset). (C) The end-stage retina in advanced atrophic AMD with geographic atrophy demonstrates extensive RPE and photoreceptor loss, with only a few photoreceptor nuclei remaining (inset). The Bruch membrane is thickened and the choriocapillaris is atrophic. *Illustration by Timothy Hengst.*

to retinal ganglion cells. Retinal ganglion cells transmit neuronal stimuli to the central nervous system via long axons that make up the optic nerve and optic tract. Other neuronal cell types in the retina such as horizontal and amacrine cells have an important role modulating and processing the light-mediated neural signaling.

The retina is an ideal tissue for using cell-based treatments. First, the transplanted cells can be visualized directly via ophthalmoscopy or a variety of noninvasive, high-resolution imaging modalities, obviating the need for a tissue biopsy. Second, the size and architecture of the retina are conducive to the transplantation of a relatively small number of cells to a specific location. This includes the surgical transplantation of cells in suspension or on sheets into the precise space or layer in which they reside, or perhaps even into the vitreous cavity that lies adjacent to the retina and is accessible via a safe, in-office injection procedure. Third, in the healthy eye the subretinal space is an

immune-privileged site with significantly diminished cellular and humoral responses [4], which may prevent rejection of foreign cells as long as the blood−retina barrier remains intact [5,6]. Fourth, there are many functional and safety end points that are noninvasive, such as visual acuity, visual field analysis, color vision, contrast sensitivity, dark adaptation, autofluorescence imaging, and microperimetry. A final advantage of the retina is that although it is a part of the central nervous system, it contains a nonsynaptic layer (the RPE) that fails as a central feature of many blinding conditions and that may render it more amenable as an early target for cellular transplantation.

Age-Related Macular Degeneration

AMD is a multifactorial degenerative disorder in the elderly that primarily affects the macula and can lead to permanent vision loss [7]. It is a major cause of vision loss in patients age 65 years and older living in developed countries, with over 2 million affected by advanced AMD in the United States alone [8]. AMD has two main clinical presentations with distinct prognostic and treatment considerations. The nonneovascular, atrophic, or dry form of AMD is associated with the deposition of subretinal lipid deposits known as drusen and progressive RPE abnormalities that can result in geographic atrophy (GA) of the RPE, choriocapillaris, and photoreceptors (Fig. 22.2B−C). The neovascular or wet form of AMD presents with more sudden vision loss owing to hemorrhage, fluid accumulation, and eventual scarring caused by the development of neovascular tissue. This form can be treated with antivascular endothelial growth factor (anti-VEGF) agents. However, anti-VEGF therapy is far from perfect; it has pharmacokinetic and compliance issues among others. No treatment exists for GA in dry AMD, although a number of immune-modulating and other potential therapies are being studied.

Both genetic and environment factors contribute to the pathogenesis of dry AMD. One of the earliest insults is oxidative stress on the RPE owing to the accumulation of lipofuscin and other by-products of the visual cycle [9]. These toxic metabolites ultimately result in RPE apoptosis with subsequent photoreceptor loss and GA. The pathogenesis is certainly multifactorial, however, with a role for choroidal ischemia and lifestyle factors such as diet and smoking. Genome-wide association studies have implicated the complement system in the pathogenesis of AMD [10,11].

As of yet, there is no established treatment to reverse or slow the progression of GA. Several pharmaceutical classes are being tested in clinical trials, including complement factor pathway inhibitors, neuroprotectants, visual cycle modulators, and immunosuppressants [12,13]. Stem cell−based therapies for end-stage dry AMD have shown promise in small safety studies [14].

Stargardt Macular Dystrophy

The most prevalent childhood-onset macular dystrophy is STGD, with an estimated prevalence of 1 in 10,000 individuals [15]. Most often it presents in the first or second decade with diminished visual acuity and the classic examination findings of bull's-eye maculopathy, pisciform flecks, and relative hypofluorescence of the choroid on fluorescein angiography. Many individuals progress to central GA with profound central vision loss. Several genes have been implicated, the most common of which is an autosomal recessive mutation in the ABCA4 gene [16]. ABCA4 is transmembrane transport protein required for the removal of all-trans-retinaldehyde from photoreceptor outer segments, and when mutated, toxic visual cycle metabolites accumulate in RPE cells.

Although this disorder has been characterized genetically and biochemically, there are no established treatments for the disease. Gene therapy trials to replace the ABCA4 gene are under way, but limitations to these trials include the size of the gene, the limited choice of nonintegrating vectors, the slow course of neural degeneration, and the fact that improvements are unlikely in advanced cases in which atrophy has already set in. Stem cell−based RPE replacement therapy has been tested in phase 1 trials with promising results in patients with advanced disease. This regenerative strategy may prove to be safe and effective earlier in the course of disease by providing the patient with dose of healthy RPE, and thus theoretically preventing further damage to adjacent macular photoreceptors and preserving central vision [14].

Retinitis Pigmentosa

Inherited retinal dystrophies have myriad clinical manifestations but ultimately result in progressive and often profound vision loss owing to photoreceptor death. These disorders affect approximately 1 in 2000 individuals and have been mapped to over 120 genetic loci thus far [17]. A subset of patients with inherited retinal degenerations

have RP, which is a clinical entity presenting with symptoms of diminished night vision (nyctalopia) and peripheral vision (visual field loss), and can progress to profound central vision loss (visual acuity loss) and even total blindness. The causative genetic mutations affect the biochemistry or structure of photoreceptors and the RPE. Rod photoreceptor dysfunction and degeneration are a hallmark of the disease, but eventually cone photoreceptors, RPE, and other cell types degenerate within the retina.

Treatment for RP and other inherited retinal dystrophies is limited or nonexistent. Except for forms caused by an underlying metabolic disorder, dietary supplements have had limited success. There has been considerable excitement regarding gene replacement therapy via viral delivery of normal copies of the mutated gene. For RPE65-associated Leber congenital amaurosis, gene delivery of RPE65 via an AAV2 vector showed success in a canine model [18] and in human patients as well [19]. There are several issues with gene therapy in RP, however. One issue is that the currently available viral vectors are not ideal, with size limits and relatively inefficient transduction efficiency. Another issue is that gene delivery is unlikely to be effective in late stages of the disease when the target cells are severely compromised or already absent. Finally, in light of the genetic heterogeneity, it would be cumbersome to create custom vectors for each form of the disease. These issues have spurred interest in cell-based therapies that could replace lost cells in advanced stages of the disease regardless of the underlying mutation.

CELL-REPLACEMENT THERAPY

The use of stem cells as a source of replacement cells to treat disease has been one of the long-held promises of the technology. For many retinal disorders such as AMD and RP, however, the degenerative process begins in one cell type but eventually engulfs adjacent cell types and layers. Treatment of end-stage disease is thus a more complex endeavor, requiring the transplanted stem cell–derived cells to rejuvenate dormant and moribund cells or even induce the genesis or migration of healthy cells into the proximity of the transplant. Treatment of end-stage disease with the goal of restoration of function, as opposed to halting disease progression, therefore may necessitate the use of multilayered, multicellular tissue transplants. Ideally, intervention could be performed early when the transplanted cells can augment the affected cells and prolong the survival of adjacent tissues. This approach carries a higher chance of success but must be reconciled with the safety and efficacy profile of the transplantation procedure early in the course of the disease when the eye is still seeing well.

The RPE is an attractive target for cell replacement therapy for both medical and societal reasons. Efforts to replace the RPE via autologous or fetal RPE transplantation go back to the 1990s, but these efforts were fraught with difficulties including insufficient cellular material and scarring at the harvest site [20–23]. In addition, because the transplantation occurred in end-stage GA lacking choriocapillaris and photoreceptors, visual recovery was unlikely to occur. The use of human embryonic and iPSCs has since risen to prominence as an attractive source of replacement human RPE. The use of stem cell–derived photoreceptors is being tested in animals and may be available for human studies in the near future.

Human Embryonic Stem Cell–Derived Retinal Pigment Epithelium

The use of hESCs to generate RPE as replacement cells has stood out as a bright spot in the field of regenerative medicine, with promising data from first-in-human transplantation trials. Although many technical difficulties had to be overcome along the way, the cellular product maintained an excellent safety and functional profile from in vitro testing to animal models to human clinical trials. hESCs are an attractive source of RPE because of their scalability and diminished immunogenicity in animal models of xenorejection [24]. However, a significant concern regarding hESC-derived RPE is that they could proliferate, form teratomas, dedifferentiate, or trigger an unremitting inflammatory response that would eliminate the transplanted cells as well as adjacent areas of functional retina.

A primary goal of preclinical and human studies thus far has been to address the safety of the transplanted allogeneic cells. These terminally differentiated hESC-derived RPE cells are amenable to extensive testing ex vivo, including for purity, cellular function, and transcriptome analysis to demonstrate strong expression of RPE markers and downregulation of hESC markers [25]. When injected beneath the retina in rodent models of retinal degeneration, the transplanted cells integrate into the RPE monolayer and improve visual function and luminance threshold response. Studies in National Institutes of Health class III immune-deficient mice showed no proliferation of the cells or spread to other parts of the body.

Schwartz et al. first demonstrated the use of hESC-derived RPE in human subretinal transplantation studies in patients with advanced GA from dry AMD and STGD [26]. Although dry AMD represents a leading cause of blindness in the developed world, patients with STGD were included in initial trials to address the role of the Bruch membrane. Senescence of the Bruch membrane in advanced dry AMD could hypothetically limit the success of this strategy, because cells were transplanted in suspension with the hope that they might survive, engraft, and polarize on the Bruch membrane. Thus, the success of this strategy may depend on the relative viability of the Bruch membrane. Therefore, younger patients with STGD and a hypothetically healthier Bruch membrane were studied as well. In this trial, the patients were given solid-organ transplant doses of immunosuppression and received a vitrectomy with subretinal injection of a suspension of hESC-derived RPE (Fig. 22.3). The investigators in this trial selected for the transplantation site a region outside the center of the macula, termed the transition zone, that was compromised but still viable and included areas of frank atrophy at the central-most aspect and normal retina at the peripheral-most aspect. This transition zone was chosen for initial transplantation for a number of reasons. First, the central macula in advanced cases of both conditions has the longest history of tissue loss including adjacent structures such as the photoreceptors, the Bruch membrane, and choriocapillaris. Thus, the central macula in advanced disease is not only long devoid of visual tissue (photoreceptors) but also devoid of blood supply (choriocapillaris), and thus far less likely to accept a safe and effective transplant. Second, this site includes areas that mimic the state of the macula earlier in the course of disease, allowing investigators to query eventual disease stage targets for RPE replacement therapy.

These phase I/II studies included nine patients with STGD and 9 with AMD. Most important, none of the patients experienced significant safety issues including nonsurgical inflammation, teratoma formation, or unwanted proliferation of the cells. Although the initial trials were meant to address safety and tolerability, 13 of 18 patients (72%) featured increased pigment at the border of the atrophic area suggestive of stem cell–derived RPE survival, and many of the patients experienced sustained improvements in visual function [14]. However, the interpretation of functional end points should be made with caution because vision was studied as a safety end point in a study with relatively few patients, lack of a formal control group other than the fellow eye, and the advanced disease present at baseline.

This initial pilot study using hESC-derived RPE delivered as a cell suspension has spurred the development of alternative treatment strategies to address the challenges inherent in this approach. These include efforts to address engraftment, polarity, confluence, maturity, and survival concerns on the senescent Bruch membrane by transplanting RPE cells grown into a confluent monolayer on a biocompatible scaffold before surgery. Although the surgical transplant procedure is necessarily more complex and potentially complicated, and the degree of biocompatibility of the scaffold will be critical, the hypothetical advantages of these approaches at the cellular level may prove to be important. At least two groups have developed scaffold-based RPE transplants derived from hESCs. One group developed a thin polyester membrane approximately 3×6 mm in size, upon which they cultured hESC-derived RPE cells [27]. This approach is already being tested in human patients with subretinal hemorrhages or RPE tears, two potentially catastrophic manifestations of neovascular AMD. Another team has begun clinical trials in patients with advanced dry AMD employing hESC-derived RPE sheets grown on thin Parylene membranes. Subretinal transplantation in a rat model of retinal degeneration demonstrated photoreceptor rescue with associated improvements in visual function [28].

The scalability and safety of hESC-derived RPE make it an attractive treatment strategy regardless of whether the cells are delivered as a suspension or as a monolayer supported on a scaffold. The main concern with hESC-derived cells, however, is the potential need for significant systemic immunosuppression; most adverse events reported in the pilot trials were directly attributable to the tolerability of systemic immunosuppression. Future studies must explore to what degree immunosuppression is necessary. An alternative approach for generating replacement RPE has been the use of autologous or human leukocyte antigen (HLA)-matched iPSCs to alleviate rejection concerns and the dangers of solid-organ transplant-dose immunosuppressive regimens.

Induced Pluripotent Stem Cell–Derived Retinal Pigment Epithelium

The ability to create RPE cells from iPSCs theoretically reduces the risk of immune rejection, but in actuality this and other critical safety and efficacy parameters may depend on the reprogramming technique and the cell type produced [24,29,30]. Regardless, iPSC-derived RPE transplantation holds enormous promise and several research efforts with pathways to human use are under way.

Many of the iPSC-based strategies use biocompatible scaffolds to deliver the RPE cells as a sheet (Fig. 22.4). A pioneering clinical trial using iPSC-derived RPE was approved in 2013 for study in Japan. In preclinical studies,

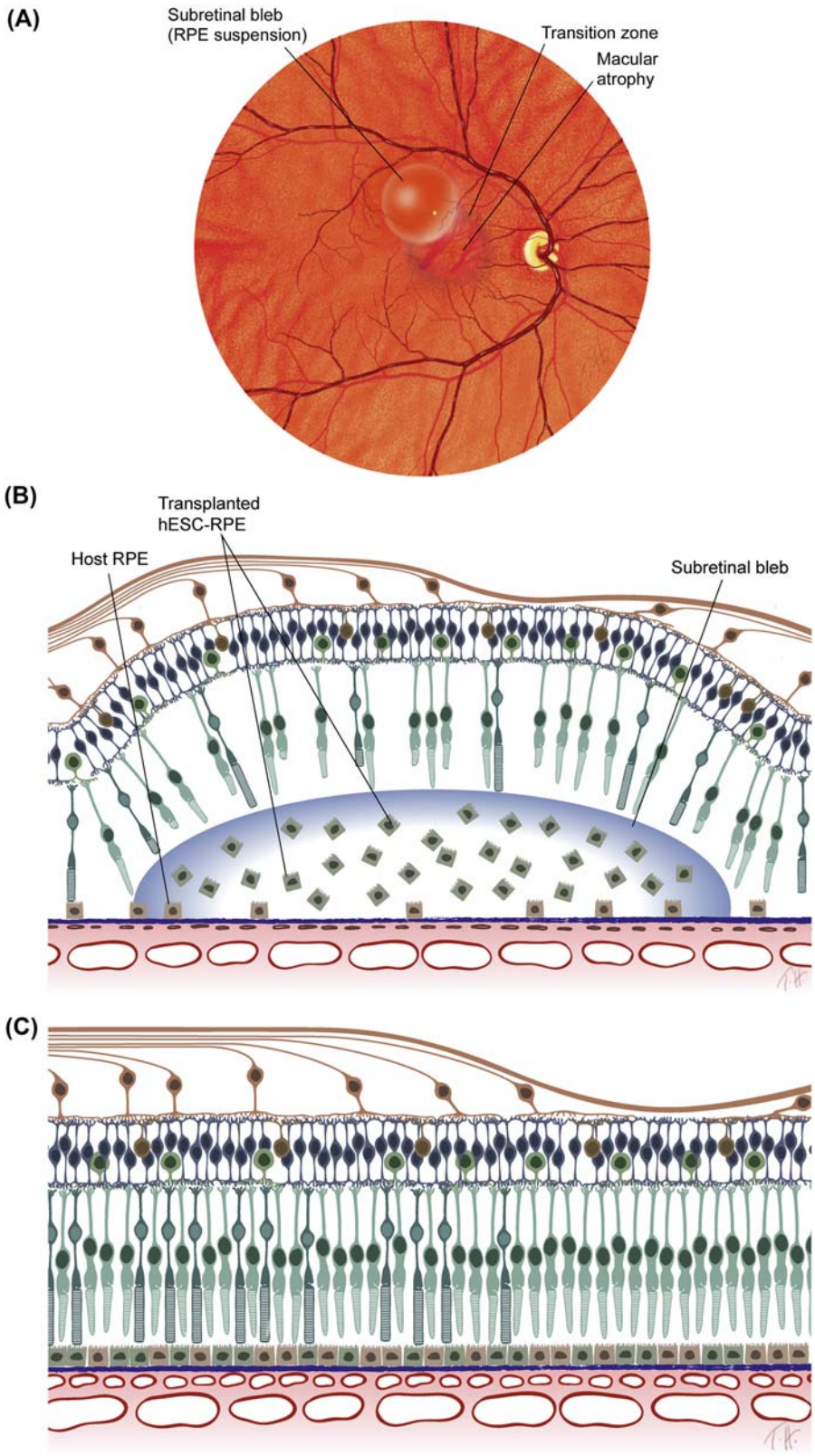

FIGURE 22.3 Schematic illustration of human embryonic stem cell–derived retinal pigment epithelium (RPE) transplanted as a suspension for macular degeneration. (A) A subretinal bleb containing a fixed volume of transplanted cells in suspension is created via a 39-gauge injection site, or retinotomy (yellow dot), in the macula. For experimental purposes, the hyperautofluorescent transition zone straddling the border of the atrophy was targeted. (B) Schematic cross-section through the bleb at the time of delivery to a diseased retina shows a suspension of transplanted human embryonic stem cell–derived RPE. (C) The ideal outcome is for the transplanted cells to survive the injection, engraft on the Bruch membrane, polarize, and then rescue or regenerate the remaining photoreceptors. *Illustration by Timothy Hengst.*

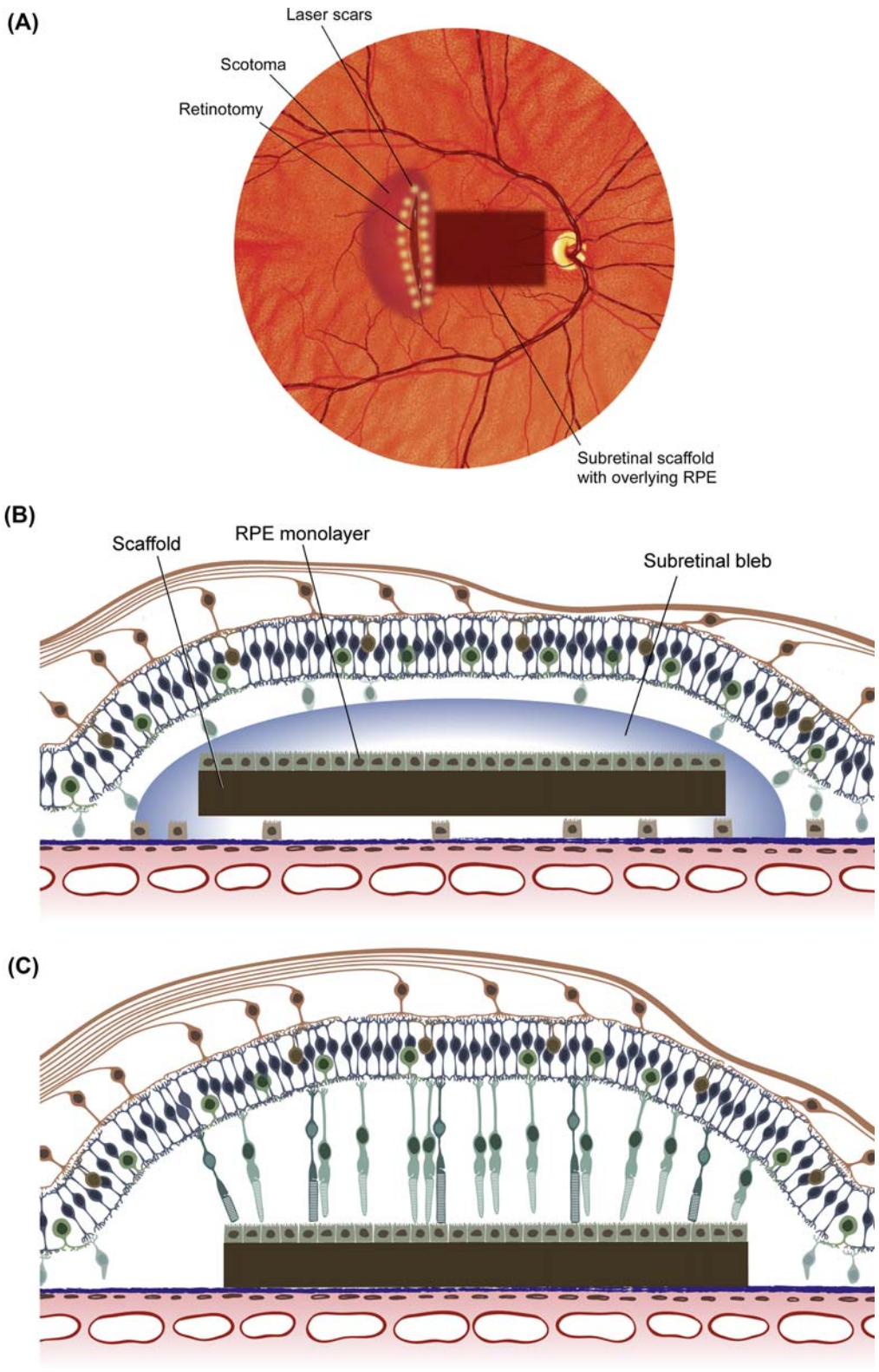

FIGURE 22.4 Schematic illustration of stem cell–derived retinal pigment epithelium (RPE) cells transplanted as a monolayer on a scaffold material. (A) The scaffold material with overlying RPE has been surgically delivered to the subretinal space. The incision in the retina (retinotomy) required to deliver the scaffold has been surgically surrounded by laser treatment to prevent postoperative retinal detachment. There is a scotoma in the nasal visual field corresponding to the crescent of injured retina including photoreceptors and ganglion cell axons. The size and thickness of the scaffold depend on design parameters. Similarly, the size of the retinotomy will vary depending on these design features and whether the scaffold and cells can be safely folded for delivery. (B) Schematic cross-section through end-stage geographic atrophy at the time of scaffold placement shows the transplanted RPE monolayer on the scaffold. (C) The ideal outcome is for the scaffold to rest directly upon the Bruch membrane and for the transplanted RPE cells to rescue the surviving photoreceptors cells. *Illustration by Timothy Hengst.*

sheets of human iPSC-derived RPE on biodegradable collagen gel scaffolds behaved like native RPE with similar gene expression profiles and functional rescue in animal models [31]. The human trials recruited patients with end-stage wet AMD and created iPSCs from skin fibroblasts. At least one patient received an autologous iPSC-derived RPE sheet, but the study was halted for safety reasons. Future work may involve the use of HLA-matched rather than autologous iPSC-derived RPE sheets owing to logistic, financial, and safety constraints.

At least two other centers are employing iPSC-derived RPE monolayers grown on a scaffold to treat GA in dry AMD. One group will use a polyester scaffold that is injected beneath the retina via a custom-designed device. Another team is developing an iPSC-derived RPE "patch" that uses a thin biodegradable poly(lactic-co-glycolic acid) (PLGA) matrix. These iPSC-RPE "patch" grafts will be delivered surgically to the subretinal space using a custom-built injector.

The use of iPSC technology to generate replacement RPE cells has the potential to mitigate the need for immunosuppressive regimens with unfavorable risk profiles, particularly in the elderly. Furthermore, the latest techniques permit the generation of xeno-free, integration-free iPSCs in clinical grade laboratory settings. Nevertheless, iPSCs are susceptible to mutations, chromosomal defects, and other transcriptional and epigenetic changes that require the implementation of stringent safety protocols. In addition, patient-derived iPSC derivatives will contain the same genetic defect, possibly precluding their use in certain disorders. However, gene-editing techniques have the potential to repair these mutations before the production of differentiated cells for autologous transplantation [32].

Scaffolds for Retinal Pigment Epithelium Transplantation

As discussed earlier, several clinical trials using stem cell–derived RPE as replacement therapy are implementing scaffold materials to deliver the cells as a monolayer. This approach has several theoretical advantages over delivery of the cells as a suspension. First, it does not require the RPE cells to engraft on a senescent Bruch membrane. Second, the cells are delivered as a monolayer, which avoids issues with cell clumping or incorrect polarization. Third, the potential is limited for the RPE cells to reflux into the vitreous cavity during surgical delivery. Despite these advantages, the delivery of RPE on a scaffold to the subretinal space requires more challenging surgical manipulations and a larger opening into the retina, both of which can result in serious vision-threatening complications. Furthermore, the biocompatibility of the scaffold material is a complex and critical issue because the scaffold must not only support cells at the time of the transplant, it must subsequently allow for physiologic processes to proceed unchecked between the RPE and the choriocapillaris. As such, the permeability of the scaffold material is critical to its success if it is not engineered to dissolve. It is still unclear which if any of these approaches will prove safe and effective, but research into scaffold materials is progressing rapidly with a wide variety of materials available.

Scaffold materials can have a broad array of characteristics including biodegradability, thickness, stiffness, and permeability. The materials themselves can be natural in the case of human donor tissue or entirely synthetic with additional surface coatings and microfabrication techniques to increase porosity or cell adhesion. Natural materials are derived from donor tissue or biologic molecules meant to simulate the properties of the Bruch membrane. These include anterior lens capsule, amniotic membrane, and biodegradable gelatin, collagen, and hyaluronate gel films. Aside from difficulties in fabricating these materials consistently, the materials are allogeneic and possibly even animal-derived, which poses immunogenicity and safety concerns. Nevertheless, the group at Riken in Japan transplanted autologous iPSC-derived RPE on a collagen gel scaffold into at least one patient [33].

Synthetic materials have a wide range of physical properties that can be leveraged to maximize RPE engraftment, permeability, and stability during subretinal delivery. Nonbiodegradable synthetic materials include Parylene, polymethyl methacrylate, and polyester. Ultrathin Parylene-C sheets (0.4 μm thick) are nonimmunogenic and mimic the permeability properties of the Bruch membrane. Sheets of hESC-derived RPE grown on micromachined sheets of vitronectin-coated Parylene have been used in pig and rat models with favorable cellular survival and safety at 3–6 months after transplantation [28,34]. An alternative material being used in human and animal studies is permeable polyester because of its strength and flexibility characteristics [27]. The biodegradable polymers such PLGA, poly(l-lactic-co-citric acid), and poly(ε-caprolactone) are potentially advantageous in that their fiber density, degradation rate, and thickness can be manipulated to achieve maximal RPE engraftment, survival, and polarization. For example, ratios of PLGA and poly(lactic acid) can be manipulated to achieve favorable porosity, degradation kinetics, and thickness, and various protein coatings such as laminins can be applied to improve RPE adhesion and survival [35]. A potential downside to these degradable scaffolds is that the materials could incite inflammation upon dissolution or that the survival of the transplanted cells would be compromised once they rest directly on the Bruch membrane. Furthermore, if any features of the scaffold become compromised during production or

transplantation, the functionality and survival of the transplanted cells may also be compromised. In essence, the scaffold itself represents a critical second variable to be considered as these programs move toward regulatory consideration.

Surgical Techniques for Retinal Pigment Epithelium Transplantation

Several surgical approaches can be employed to deliver the RPE cells. The procedure to deliver the cells must be carried out with acceptably low risk to the eye, regardless of the safety and efficacy of the transplanted cells themselves. For the delivery of RPE cells to the subretinal space, the least challenging surgical technique is injection of a cellular suspension via a subretinal cannula. This approach was used in the initial phase I/II studies of hESC-derived RPE for AMD and STGD [26]. It requires a standard three-port pars plana vitrectomy to be performed followed by insertion of a 38-gauge subretinal cannula through the retina (retinotomy) to deliver the cells into the subretinal space, raising a subretinal bleb. After the procedure, the bleb slowly flattens over a few hours as the fluid is absorbed; it is hypothesized that the RPE cells that survive the procedure may subsequently engraft and polarize. Theoretical surgical advantages to this approach are that the small retinotomy used to inject the cells seals spontaneously with little or no risk of subsequent retinal detachment and no discernible secondary scotoma formation from disruption of retinal tissue, and that it relies on skills with which most vitreoretinal surgeons are comfortable.

An alternative, more complex surgical approach must be used when delivering RPE sheets grown on a scaffold material. For the RPE sheet to be delivered in an atraumatic fashion, a large retinotomy must be made adjacent to the macula and a specialized delivery device must be employed to inject the scaffold. The size of the retinotomy depends on the dimensions of the scaffold and whether the material is foldable, but in general this will require the use of laser photocoagulation combined with a tamponade agent such as silicone oil to seal the retinal incision at the end of the surgery to keep the retina attached. Therefore, these techniques come with an increased risk for retinal detachment and a larger, potentially clinically significant scotoma associated with the retinotomy site and surrounding laser scars. Other potential downsides include the possibility the scaffold could be implanted upside down or that the cells slough off during insertion, although these risks should be mitigated with a short surgical learning curve.

A third method for subretinal delivery of cells is *ab externo* via a cannula that passes through the subretinal or suprachoroidal space. These techniques were pioneered in a trial studying delivery of human umbilical tissue-derived cells to the macula. (Note that these cells were not differentiated into retinal cell types but were instead intended to serve as neuroprotective cytokine-secreting cells.) This was necessary because initial trials using a standard vitrectomy with subretinal injection of a cellular suspension led to severe proliferative vitreoretinopathy (scarring on the retinal surface with subsequent contraction and complex retinal detachment) if any cells were exposed to the vitreous cavity. Thus, this alternative approach theoretically avoids the vitreous cavity by performing a scleral cutdown followed by insertion of a microcatheter into the subretinal space. However, the technique was plagued by difficulties including an inability to control the cannula tip and the creation of inadvertent retinal perforations. The refined methodology involves threading the cannula through the suprachoroidal space toward the macula. A microneedle is then extended under direct visualization into the subretinal space and the cells are delivered. This approach is promising but requires novel, nonstandard surgical techniques.

Regardless of the technique used, the transplant site must be carefully chosen on a case-by-case basis. The first patients to undergo hESC-derived RPE transplants received the cells in the peripheral macula owing to the large area of central atrophy and to avoid detaching the fovea. Subsequent transplantations occurred at the transition zone between the atrophic retina and the relatively healthy retina, an area with compromised but still viable RPE and photoreceptors. The preferred transplantation site for RPE cells on a scaffold may be within an area of atrophy, but RPE survival and function are questionable given the extent of photoreceptor and choriocapillaris loss [36]. Further study is necessary to determine where on the spectrum of disease the ideal candidates for cellular therapy will reside, although it seems likely that treating patients earlier in the course of disease will prove beneficial.

Photoreceptor Transplantation

Photoreceptor cell transplantation represents a possible treatment strategy for retinal dystrophies such as RP. As for RPE transplantation, the use of stem cell–derived photoreceptors to replace lost cells is advantageous in that it can be used in advanced cases and across many forms of the disease, regardless of the underlying genetic defect. However, the transplanted photoreceptor cells must integrate into the photoreceptor layer, form appropriate synapses, and extend outer segments toward the apical processes of the RPE. These requirements may be hampered by

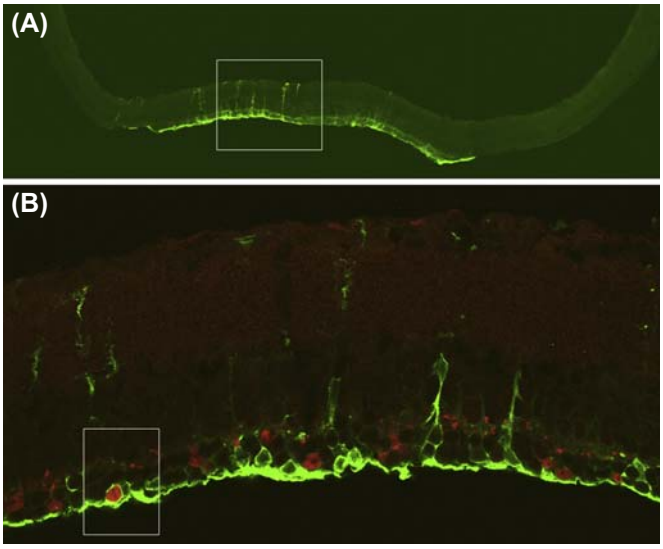

FIGURE 22.5 Integration and survival of transplanted human embryonic stem cell–derived retinal cells in Crx-deficient mice, a model of retinal degeneration. The transplanted cells (green) are found in the outer retina and some express recoverin (red), a photoreceptor marker. *Reprinted from Lamba DA, Gust J, Reh TA. Transplantation of human embryonic stem cell-derived photoreceptors restores some visual function in Crx-deficient mice. Cell Stem Cell 2009;4(1):73–9, Copyright (2009), with permission from Elsevier.*

ongoing cellular dysfunction, apoptosis, and inflammation in the disease state. As is the case for RPE transplantation, early intervention may provide the best opportunity for favorable anatomic and functional outcomes.

Several groups have published encouraging results in preclinical animal models of RP. MacLaren et al. demonstrated that mouse RPCs can be transplanted successfully into a mouse model of rod degeneration with the restoration of synaptic connectivity and light sensitivity [37]. The limited availability of human retinal tissue precludes the use of this approach in human clinical trials, but hESC-derived retinal progenitors can also integrate into the photoreceptor layer, form synapses, and partially rescue visual function in a mouse model of early-onset RP (Fig. 22.5) [38]. Investigators have migrated toward using photoreceptor precursors derived from three-dimensional rather than two-dimensional culture methods as a way to enhance the developmental verisimilitude of the transplanted cells [39]. In addition, sheets of developing three-dimensional retina cut out from iPSC-derived optic vesicle-like structures have been transplanted beneath the retina in diseased mice with evidence of photoreceptor integration and functional improvement [40,41].

Although efforts to transplant photoreceptors lag behind the RPE, advances in culture methods and promising results in animal models will make human photoreceptor transplantation studies a reality. Whether the transplanted cells can be delivered in sufficient quantities to improve visual function significantly and delay or even halt ongoing cellular loss in the disease state remains to be seen [42].

CELL-BASED NEUROPROTECTION

In addition to serving as a source of replacement cells, stem cells are being studied for neuroprotection and neurotrophic support. This strategy relies on the transplanted cells to secrete neurotrophic factors that halt ongoing pathologic processes including apoptosis and thus sustain the surviving tissues. A potential advantage to this approach is that the final cell product does not require the production of terminally differentiated cells from pluripotent stem cells. For example, bone marrow–derived hematopoietic stem cells can be harvested from the patient, purified, and transplanted in an autologous fashion without the need for systemic immunosuppression. Even allogeneic transplantation of other stem cells such as umbilical tissue-derived stem cells may not require immunosuppression. Furthermore, because the cells are functioning via a paracrine effect, it may be sufficient to deliver the cells in proximity to the target tissue, such as the vitreous cavity. Potential disadvantages to this approach include local off-target effects such as proliferative vitreoretinopathy and the need for repeated dosing.

Bone Marrow–Derived Stem Cells

Traditionally, two types of bone marrow–derived stem cells (BM-SCs) have been described (mesenchymal and hematopoietic) and both are being tested to treat retinal disease [43]. These cells must be harvested by bone marrow aspiration but they can be then used in an autologous fashion. Whether these transplanted cells integrate and differentiate into retinal cell types has not been clearly established, and it appears that the benefit more likely derives from the paracrine effect of secreted cytokines [43]. Hematopoietic $CD34^+$ stem cells are multipotent adult stem cells capable of self-renewal; they have been extensively characterized for use in hematologic disorders. Studies in several rodent models of retinal degeneration have shown a protective effect of human adult hematopoietic stem cells delivered via intravitreal injection [44,45]. The other bone marrow cell type, the so-called mesenchymal stem cells, have elicited controversy because this cell population may not have stem cell properties and instead represents committed multipotent progenitor cells [46]. Mesenchymal cells have been delivered beneath the retina with favorable initial results in animal models [47,48].

Anatomic and functional improvements observed in these animal models of retinal degeneration have spurred various sites to initiate clinical trials in humans using BM-SCs. Initial experience with autologous $CD34^+$ BM-SCs in human patients with RP demonstrated no adverse effects from the intravitreally delivered cells [49]. A subsequent study in patients with a variety of retinal conditions including retinal vascular disease, AMD, and inherited retinal disorders also showed no detrimental effects of the cells [50]. Convincing evidence is lacking that the cells provide a functional and anatomic benefit, however.

Umbilical Tissue-Derived Stem Cells

hUTCs are isolated from digested human umbilical cord tissue and can be tested extensively before use. Although they are allogeneic, there is evidence the cells may be minimally immunogenic with no expression of major histocompatibility complex (MHC) class II and lower expression of MHC class I [51]. The therapeutic benefit is thought to derive from the strong extracellular secretion of several trophic cytokines such as brain-derived growth factor, hepatocyte growth factor, and glial cell-derived neurotrophic factor [52]. Subretinal delivery of hUTCs in a rodent model of retinal degeneration led to improvements in visual function compared with untreated animals [53].

The functional benefit of hUTCs in AMD or RP remains to be established, but human trials are underway. The initial pilot study in patients with advanced RP delivered the hUTCs to the subretinal space via pars plana vitrectomy, but this approach was abandoned owing to concerns for epiretinal membrane formation and proliferative vitreoretinopathy. Follow-up studies are instead using a novel *ab externo* delivery system in patients with advanced dry AMD.

Neural and Retinal Progenitor Cells

Neural progenitor cells (NPCs) and RPCs are being evaluated as a treatment for atrophic AMD and photoreceptor degenerations such as RP. Both cell types are sourced from human fetal tissue, which may raise ethical concerns. In a rat retinal degeneration model, subretinally delivered NPCs persisted for months and improved visual function remarkably [54]. Transplanted mouse RPCs may even integrate and differentiate into photoreceptor-like cells in the correct retinal layer with expression of photoreceptor markers [55]. Human clinical trials using NPCs and RPCs are under way, and it may be possible in future trials to generate these cells from pluripotent stem cells [56,57].

DISEASE-IN-A-DISH MODELING FOR RETINAL DISORDERS

Efforts to treat retinal disease with cellular therapy represent an important application of stem cell technology. Some of these same techniques can also be used to model human retinal disorders in vitro in a patient-specific manner and even serve as a platform for testing customized therapeutic agents. Stem cells derived from skin fibroblasts or peripheral leukocytes can be differentiated into various retinal cell types and can even be coaxed into forming three-dimensional optic vesicle-like structures with a multilaminar architecture reminiscent of the human fetal retina.

Induced Pluripotent Stem Cell–Based Phenotyping

Traditionally, the study of human disease has occurred in animal models to allow for detailed molecular, genetic, cellular, and developmental analysis. However, it is common for animal models of retinal disease to recapitulate the human state only partially, or in some cases fail to do so entirely [58]. Human iPSCs serve as a promising tool to model disorders of the RPE and other retinal cell types [59]. This approach not only eliminates issues with cross-species differences and the expense of laboratory animals, it allows for the creation of patient-specific cell lines containing the precise genetic mutation in the same genomic milieu.

The first "disease-in-a-dish" model for a retinal disorder was created using iPSCs from patients with RP [60]. In this study, five iPSC lines were created from five patients with distinct causative mutations. Photoreceptors differentiated from these lines showed accelerated degeneration compared with controls, and application of the antioxidant vitamin E had a differential effect depending on the causative gene. This demonstrated the utility of patient-derived iPSC lines for modeling human disease and testing therapeutics in a customized fashion. Another study created iPSC-derived RPE cells from patients with Best vitelliform macular dystrophy [61]. Photoreceptor outer segment degradation was impaired in these RPE cells, leading to oxidative stress. Furthermore, iPSC-derived cells can be used to verify the pathogenicity of previously undiscovered mutations. This approach has been used to determine the pathogenic mechanism of novel disease-causing variants, such as a cryptic splice site mutation in the RPE65 gene [62] and a splice site mutation in USH2A [63].

Disease-in-a-dish modeling has also been employed to validate a variety of treatments such as small molecule therapeutics, gene therapy, and gene editing. iPSC-derived RPE cells have been subjected to chronic oxidative stress to simulate AMD [64] and then treated with various compounds that reduce oxidative stress [65]. A similar approach was used to test the effects of vitamin B6 supplementation in gyrate atrophy [66] and inhibitors of endoplasmic reticulum stress in RP [67,68].

Gene delivery via viral vector to patient-derived iPSC lines can also be used in preclinical studies, especially when animal models do not recapitulate the phenotype. Choroideremia is an inherited condition resulting from mutations in the REP1 gene that lead to degeneration of the choriocapillaris, RPE, and retina. The mouse knockout is lethal, but viral gene delivery rescued REP1 function in iPSCs derived from patients with choroideremia [69]. This approach has also been used in several other iPSC-based disease models including MFRP-mutant RP [70], CEP290-mutant Leber congenital amaurosis [71], and CLN3-mutant Batten disease [72].

Genome engineering represents a promising avenue to augment these treatment strategies [73]. Gene editing can correct a specific mutation in situ so that the wild-type gene is expressed under the endogenous promoter and avoids issues with size limits on genes delivered via viral vector. This rapidly evolving technology has been applied in two studies that show the feasibility of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 gene targeting in rodent eyes via intravitreal [74] and subretinal delivery approaches [75]. Eventually, it may even be possible to differentiate gene-edited iPSCs into a particular retinal cell type and then transplant the differentiated cells autologously in humans.

Three-Dimensional Retinal Organoids

The ability to generate complex human tissues that resemble organs in vitro has generated excitement within the field of regenerative medicine [76]. Eiraku et al. first demonstrated the capacity of mouse ESCs to form self-organizing optic vesicle-like structures containing all major cell types of the retina including photoreceptors, bipolar cells, and ganglion cells in a laminar arrangement [77]. This landmark publication set the stage for future refinements including the use of human iPSC (Fig. 22.6) [78,79], the demonstration of beautifully arranged functional photoreceptors [80], and technique modifications to accelerate the process [81]. Despite these advances, the process of culturing human retinal organoids can be labor-intensive and produce variable results and has yet to recapitulate important components of the retina including the optic nerve and vessels, for example.

Besides their use in the modeling of human retinal development and disease, three-dimensional retinal organoids may also be used as a source of replacement cells for diseases such as dry AMD and RP. It is possible that the generation of replacement cells in this fashion may provide more biologically authentic cells because their development occurred in a three-dimensional environment adjacent to other neuroretinal cells. Rod photoreceptors generated via a three-dimensional culture protocol have been dissociated and then injected beneath the retina in a mouse model of rod degeneration with evidence of functional integration into the host retina [39]. Another approach is to transplant entire sheets of stem cell–derived retinal tissue. Mouse ESC- and iPSC-derived retinal sheets transplanted into an RP mouse model can reconstitute the photoreceptor layer and form synapses in the outer plexiform layer [41]. There is also evidence these transplanted sheets elicit light-dependent behaviors and electrophysiologic responses [40].

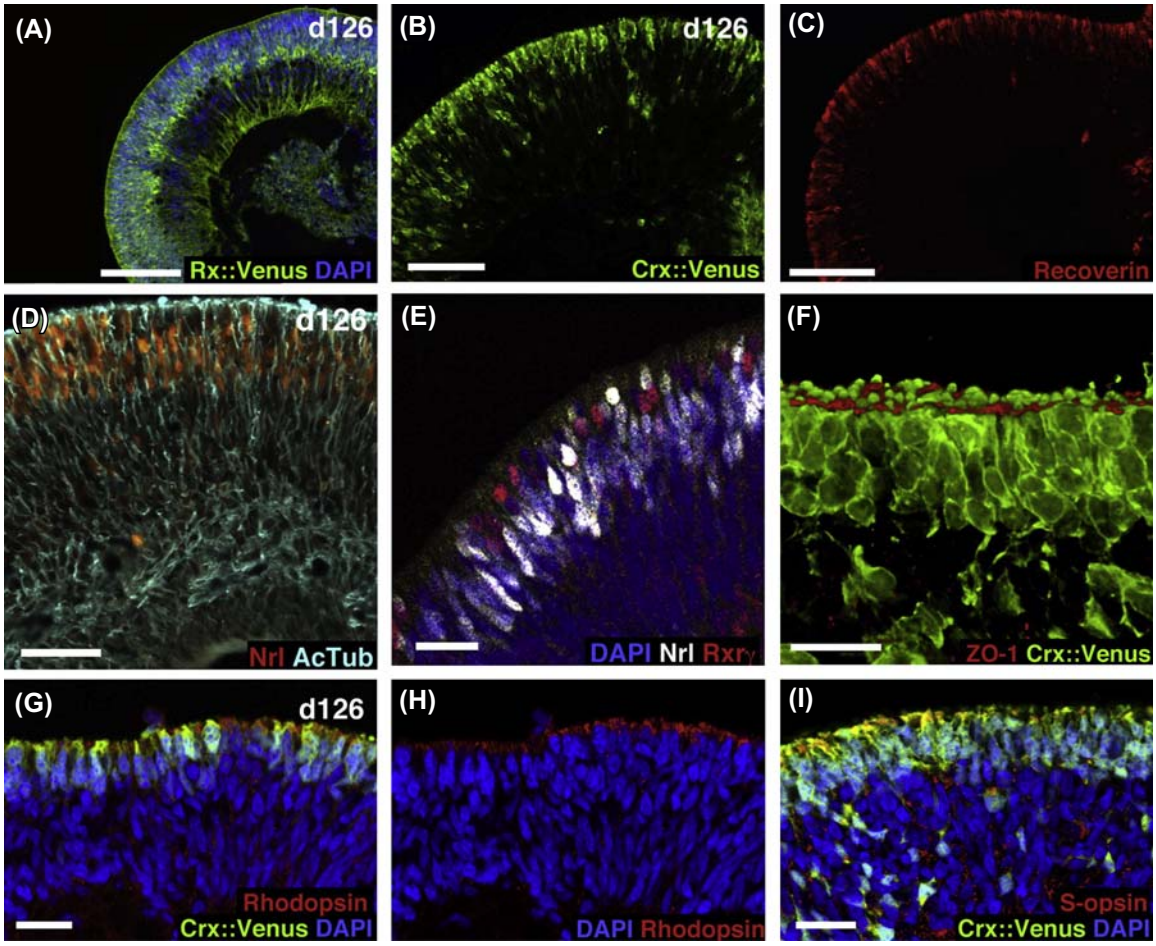

FIGURE 22.6 Self-organizing optic vesicle-like structures derived from human embryonic stem cells display remarkable complexity. The neuroretinal epithelium is multilayered and contains densely packed photoreceptors (recoverin$^+$) along the apical side. Rods (Nrl$^+$) and cones (Rxr-γ^+) are present with ZO-1$^+$ apical junctions. The visual pigments rhodopsin and S-opsin are expressed along the apical surface, but outer segments do not form. *Reprinted from Nakano T et al. Self-formation of optic cups and storable stratified neural retina from human ESCs. Cell Stem Cell 2012;10(6):771–85, Copyright (2012), with permission from Elsevier.*

CONCLUSION

Research in regenerative medicine is accelerating at a remarkable pace owing to the exceptional promise it holds for understanding and potentially treating a variety of medical disorders, many of which are currently untreatable. This is especially true for diseases of the retina. The next revolution in retina may be driven by novel gene and cell delivery approaches, both of which benefit from advances in stem cell technology.

The ideal treatment is one that restores the anatomical and functional integrity of the retina with minimal risk to the eye and to the patient. Although advances have been made and many more are imminent, there is much work to be done. The use of systemic immunosuppression carries significant risk, especially in the elderly population. Surgical delivery techniques need to be refined to minimize iatrogenic complications. Autologous iPSC-derived cells may reduce the risk of rejection, but they are costly and associated with a risk of mutations and chromosomal anomalies. Some of these issues may be resolved by ongoing initiatives such as the creation of HLA-matched iPSC banks and optimizing scaffold materials to simplify the surgical delivery. Nevertheless, other hurdles remain, such as the reconstitution of synaptic connectivity after cell replacement and the functional recovery of end-stage GA in which multiple layers have degenerated.

The future of stem cell–based treatments in the eye will rely heavily on tissue engineering approaches, such as three-dimensional microfabrication of scaffolds populated with various retinal cell types in a laminar arrangement. This could include the creation of a capillary network populated with iPSC-derived endothelial cells [82]. Many of these treatments could emerge from improvements in cell differentiation protocols and retinal organoid technology.

Despite the obstacles that lie ahead, there is substantial optimism for cell-based therapies in the retina. Several clinical trials using stem cell−derived treatments are either under way or due to begin within the next few years. The ability to recapitulate human retinal development in culture using patient-derived pluripotent stem cells may also generate deep insights into the pathophysiology of retinal disease. It is hoped that these advances will not only provide cures for untreatable blindness but also lead the way for neurodegenerative disease and the entire field of regenerative medicine.

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CHAPTER

23

Stem Cells for Traumatic Brain Injury

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INTRODUCTION

Epidemiology

Traumatic brain injury (TBI) is a leading cause of death and disability in the United States. Approximately 1.5 million people experience a TBI yearly in the United States. The Centers for Disease Control and Prevention (CDC) estimate that 275,000 people are hospitalized annually as a result of a TBI. The yearly TBI mortality rate exceeds 50,000 and it is responsible for 30% of all injury-related deaths [1]. In addition, TBI is the leading cause of death in children and adolescents. Surviving patients have varying levels of long term-sequela. Overall, 6.5 million patients are burdened by the physical, cognitive, and psychosocial deficits associated with TBI, leading to a total economic impact of approximately $76.3 billion in medical costs and lost wages [2].

The CDC collects data on TBI mechanisms of injury and trends in patient outcomes. Fig. 23.1 illustrates the leading causes of TBI in the United States from 2006 to 2010. Falls were the leading cause of TBI, accounting for 40% of all

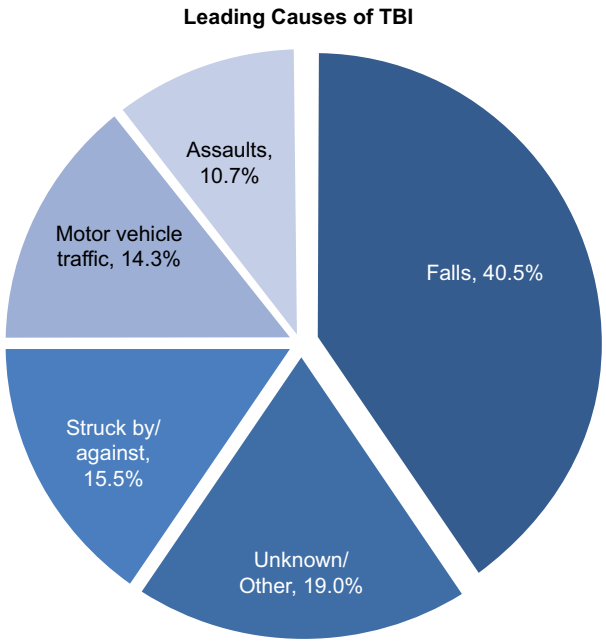

FIGURE 23.1 Centers for Disease Control and Prevention (https://www.cdc.gov/traumaticbraininjury/get_the_facts.html) pie chart demonstrating the most common causes of traumatic brain injury (TBI) in the United States from 2006 to 2010. Falls were the leading cause, followed by unintentional blunt trauma, motor vehicle accidents, and assaults.

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injuries that resulted in an emergency department visit, hospitalization, or death. Unintentional blunt trauma (being hit by an object) was the second leading cause, accounting for 15% of TBIs, followed by motor vehicle accidents, which made up 14%, and finally, assaults at 10%. TBI-related emergency department visits increased by 70% from 2001 to 2010. However, the death rate during this period decreased by 7%, largely owing to postinjury supportive care that focuses on modulating intracranial pressure (ICP) and cerebral perfusion pressure (CPP). Unfortunately, this medical management of TBI is still merely supportive care that focuses on preventing further damage without the ability to restore function.

Classification of Traumatic Brain Injury

Development of successful TBI treatments requires a clear classification of brain injury severity. Multiple clinical scoring systems have been used to classify TBI. The Glasgow Coma Scale (GCS) has been adopted to assess TBI severity and is now one of the most used scoring systems. The GCS gives a clinical score based on the patient's ability to open his or her eyes, communicate, and follow motor commands. The scale ranges from 3 to 15; GCS scores of 3–8 are classified as severe TBI, scores of 9–12 are classified as moderate TBI, and scores of 13–15 are classified as mild TBI. The abbreviated injury scale and injury severity score are additional criteria to stratify TBI based on anatomic injury, which are often available only late into a clinical course.

Several imaging classifications have been developed as an adjunct to scoring TBI severity. The Marshall classification of TBI is a computed tomography (CT) scan-derived prognostic metric that places patients into one of six categories of increasing severity. The scoring system is primarily concerned with the degree of swelling (midline shift and/or compression of basal cisterns) and the presence or size of contusions and hemorrhages. It has been able to predict both the risk of increased ICP and outcomes in adult patients with TBI [3]. Limitations of the Marshall classification system include difficulty categorizing patients with multiple injury types and standardization of certain features of the CT scan [4]. The more recently developed Rotterdam CT score of TBI includes four independently scored elements: basal cistern compression, midline shift, epidural hematomas (EDH), and intraventricular blood or traumatic subarachnoid hemorrhage (SAH) [5]. The Rotterdam scoring system may become a more preferable imaging measure of TBI severity after further validation.

Identifying biomarkers for TBI could have many potential applications in the way TBI is diagnosed and treated. A point of care test could identify TBI early and assist in the initial stages of management. Other biomarkers could serve to highlight the specific neuropathophysiology behind the injury or even injury severity. With regard to stem cell therapeutics for brain injury, a biomarker could be used in the acute and chronic stages of TBI to follow the response to different therapeutic interventions. A literature review performed in 2016 examined 12 biomarkers (S100β, glial fibrillary acidic protein [GFAP], ubiquitin C-terminal hydrolase-L1, neuron-specific enolase, neurofilaments, myelin basic protein, spectrin breakdown products, Tau, microtubule-associated protein 2, amyloid β, cytokines such as tumor necrosis factor-α (TNF-α), and interleukin (IL)-6, and autoantibodies against brain proteins such as GFAP) that have been studied to diagnose TBI [6]. The review found that nearly all 12 biomarkers were rapidly elevated after injury and may serve as a diagnostic tool. Although not specific for TBI, the negative predictive value of the biomarkers could be a clinical tool to rule out a brain injury. Although the clinical use of biomarkers in TBI is still in its infancy, the potential for broad clinical application is promising and many clinical trials are ongoing.

PHASES OF BRAIN INJURY

Primary Versus Secondary Brain Injury

TBI is divided into two distinct phases: "primary" and "secondary" brain injuries. The primary injury occurs at the time of the trauma, when energy is dissipated within the substance of the brain, either from a closed blunt injury or a penetrating wound. The primary injury is irreversible and includes direct neuronal, glial, and vascular disruption. The primary injury may be caused by contusions, shear injuries, or compression from hemorrhage. Contusions can occur under fracture sites, the point of impact (coup contusion), or at regions distant from the initial impact (contrecoup contusion). Shear injury, often referred to as diffuse axonal injury, occurs when axons tear as the brain shifts and rotates inside the bony skull. Hematomas and hemorrhages are classified by location within the brain. EDH are often caused by temporal bone skull fractures that lacerate the middle meningeal artery. EDH can develop into large, lens-shaped, extraaxial hematomas that compress the brain parenchyma and increase ICP. Subdural hematomas are found between the dura and arachnoid mater from tearing of the bridging veins in this subdural space. Large

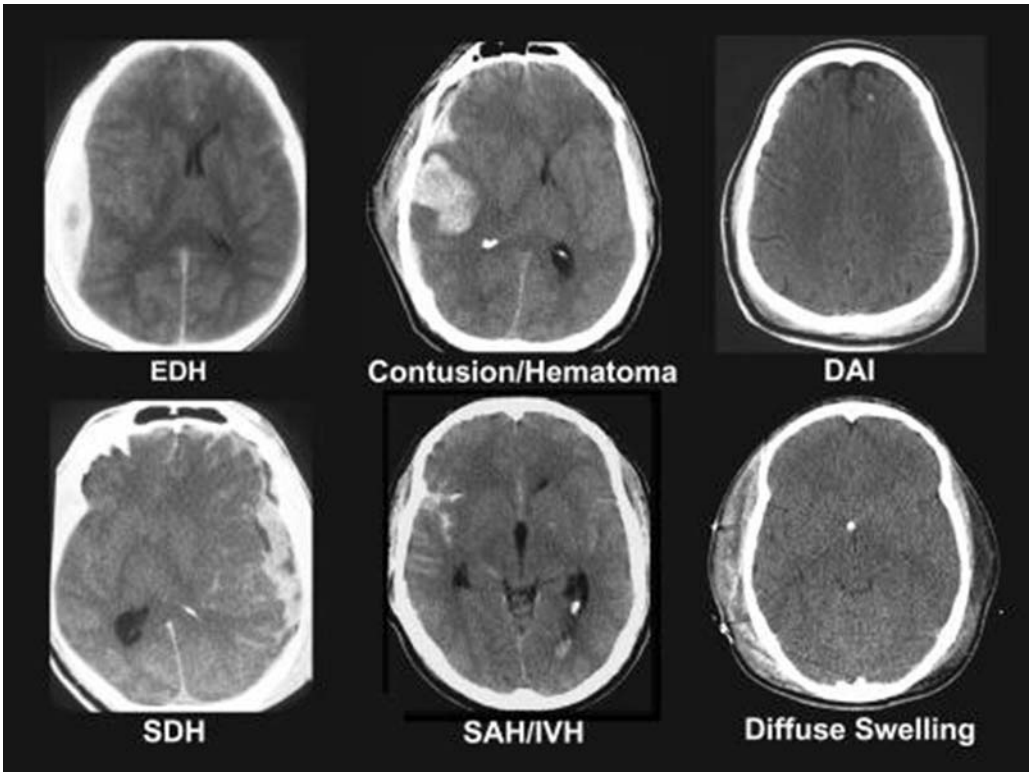

FIGURE 23.2 Different types of traumatic brain hemorrhages. *DAI*, diffuse axonal injury; *EDH*, epidural hematoma; *IVH*, intraventricular hemorrhage; *SAH*, subarachnoid hemorrhage; *SDH*, subdural hematoma.

crescent-shaped blood collections can lead to an elevated ICP and uncal herniation. SAH is bleeding between the arachnoid and pia mater. Patients with SAH can develop cerebral vasospasm, which worsens further ischemic injuries (Fig. 23.2).

The second phase of TBI begins hours after the trauma and can last decades after the initial injury [7]. Secondary injuries are the result of biochemical events that may accelerate initial cellular injury or cause new injury altogether [8,9]. The effects of secondary brain injury are caused by increased neuronal excitotoxicity, disturbances in ionic homeostasis, neuronal apoptosis, and the initiation of inflammatory and immune responses. Secondary injury mechanisms are cumulative and create a toxic local environment around the site of injury. The resulting increase in blood–brain barrier (BBB) permeability and the ensuing cerebral edema serve to amplify the neuroinflammatory signal by exacerbating the initial ischemic injury.

Neuroinflammation

TBI increases neuronal excitotoxicity and prompts disturbances in ionic homeostasis that contribute to secondary neuronal cell injury. Both human and animal models have found elevated levels of the excitatory amino acid glutamate in the brain after TBI [10,11]. Increased glutamate levels lead to neuronal excitotoxicity and a resulting hypermetabolic state. Positron-emission tomography (PET) imaging in patients with severe TBI found hyperglycolysis regionally at the site of injury and diffusely throughout the cerebrum [12]. Hyperglycolysis leads to the depletion of neuronal cell adenosine triphosphate stores and resultant disturbances in ionic homeostasis resulting from active ion pump failure. Electrochemical equilibrium across the neuron is lost owing to inactivation of the Na^+/K^+ pump and activation of ion channels by the increased glutamate levels. A massive intracellular influx of calcium occurs leading to calpain-mediated spectrin proteolysis triggering increased levels of membrane breakdown and axonal injury. These pathways result in the sustained secretion of proinflammatory cytokines and cytotoxic molecules from the damaged neurons. Fig. 23.3 demonstrates elevated intracerebral proinflammatory cytokines identified in specific areas and at specific time points relative to a TBI carried out in a rodent model using a controlled cortical impact (CCI) injury [13].

Central to the secondary inflammatory responses to TBI are microglia, the macrophages of the central nervous system (CNS) [14]. Consistent with their role as CNS macrophages, microglia are responsible for clearing dead cells

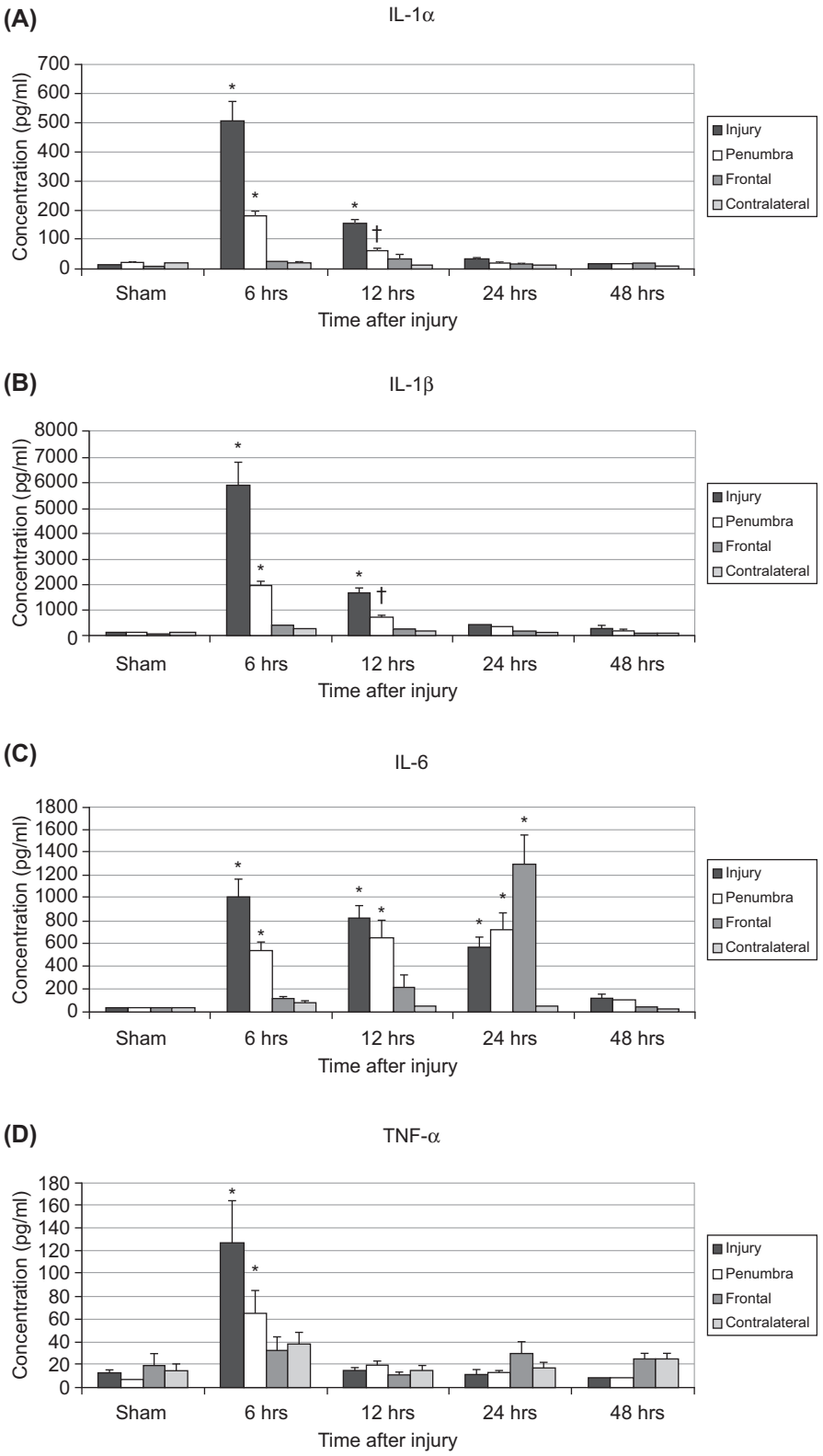

FIGURE 23.3 Elevated intracerebral cytokines identified in specific areas and at specific times relative to the traumatic brain injury (TBI). The proinflammatory cytokines interleukin (IL)-1α (A), IL-1β (B), and IL-6 (C) and tumor necrosis factor (TNF)-α (D) were significantly elevated 6 hours after controlled cortical impact in the injury and penumbral regions compared with sham animals (*$P < .01$ for all). IL-1α, IL-1β, and IL-6 remained elevated through 12, 12, and 24 h, respectively (*$P < .01$ or $^{\dagger}P < .05$). In the frontal area, IL-6 was significantly increased at 24 h (33- to 50-fold, $P < .01$, Dunnett test), but not at 6 or 12 h after TBI.

and other debris such as astrocytes and myelin. Under homeostasis, microglia are highly mobile and provide continuous surveillance of their cellular milieu (M0-phenotype) [15,16]. The hostile local milieu that results from the proinflammatory molecules during the secondary phase of TBI causes microglia to differentiate into a neurotoxic, proinflammatory state (M1-like phenotype). M1-like microglia worsen secondary neuronal cell injury by perpetuating the production of proinflammatory cytokines and cytodestructive substances. Moreover, M1-like microglia upregulate chondroitin sulfate proteoglycans, which are powerful inhibitors of axon regrowth [17,18]. Animal models have shown that within 1 week of a brain injury, there is a phenotypic shift toward the M1-like microglial response [19]. The ratio of M1-like (proinflammatory) to M2-like (antiinflammatory) microglia can be attenuated with stem cell therapy, which leads to improvements in cognitive behavior [20]. Fig. 23.4 illustrates a TBI that resulted in chronic activation of microglia that was attenuated with multipotent adult progenitor cell (MAPC) treatment (CCI-10) in the dentate gyrus. Similarly, Zanier et al. demonstrated that human bone marrow mesenchymal stem cells (MSC) infused into brain-injured mice promoted an M2-like polarization of microglia. This favorable dichotomization resulted in M2-like mediated repair responses and long-term neurologic recovery [21].

TBI also elicits a dramatic rise in the number of infiltrating macrophages at the site of injury. The pathways responsible for macrophage infiltration are still not well-understood, but they may deal with the binding of C−C-chemokine receptor 2 (CCR2). Hsieh et al. used a CCI injury model to demonstrate a greatly reduced number of infiltrating macrophages (about 80%−90%) after TBI in mice that were CCR2-deficient compared with wild-type animals. CCR2-deficient mice showed improved functional recovery (spatial learning/memory) and neuronal survival, although there was no difference in the volume of tissue loss [22]. Other preclinical works have shown that infiltrating macrophages activate microglia by releasing proinflammatory cytokines such as IL-1 and TNF-α [23,24]. In the absence of infiltrating macrophages, there is a reduction in microglial activation [25] and therefore a reduction in neuroinflammation. These discoveries have important implications for progenitor cell therapy for TBI and also question the inflammatory roles of the different myeloid lineages. The results of these studies could lead one to postulate whether infiltrating macrophages are proinflammatory and resident microglia are antiinflammatory until provoked by these hostile invaders. In this scenario, stem cell therapy would have a stronger role in downregulating the peripheral macrophage response to TBI, possibly as demonstrated through their effect on the reticuloendothelial system.

Assessment of microglial activation patterns in vivo may soon serve as a marker of TBI severity, as demonstrated by an in vivo proinflammatory response to CCI using PET/CT imaging with the PET radioligand [^{11}C]PBR28 [26]. Peripheral benzodiazepine receptors, also known as the translocator protein, are upregulated on the mitochondrial membranes of activated microglia (M1-like). Uptake of the radioligand [^{11}C]PBR28, as measured by standardized uptake values (SUV), was increased in a dose-dependent manner based on the severity of the TBI, as seen in Fig. 23.5. These SUV findings were confirmed ex vivo by quantifying the cellular morphology of activated (M1-like) versus semiactivated versus nonactivated microglia (M2-like) in thalami of the same rodents. Similar to the in vivo results, the most severely injured animals had the highest numbers of activated microglia (M1-like), followed by the moderately injured animals, and finally the sham group, as seen in Fig. 23.6. Future experiments are being planned to determine the utility of the radioligand [^{11}C]PBR28 as a marker of stem cell therapeutic efficacy after TBI in a rodent model.

Blood−Brain Barrier Permeability

The BBB is an anatomical structure that controls the flow of molecules in and out of the CNS. Cells that contribute to the structure of the BBB include endothelial cells and tight junction proteins. The opening and closing of tight junction proteins regulate transport of essential molecules and maintenance of a stable environment. There is communication between the cells of the BBB and the cells of the CNS, which include astrocytes, neurons, and microglia. Interaction between cells of the BBB and CNS has an important role in the control of cerebral blood flow [27,28].

TBI opens the BBB, allowing progression of the neuroinflammatory response. In addition, breakdown of the BBB after TBI increases microvascular permeability, exacerbating vasogenic cerebral edema with concomitant elevations in ICP. Shortly after TBI, an array of effector cells, including neutrophils and monocytes, migrate from the peripheral blood to the site of injury [29]. Infiltrating macrophages can further amplify microglia activation into the neurotoxic, proinflammatory phenotype. In the absence of closure of the BBB, macrophage infiltration is left unchecked and contributes to a prolonged inflammatory response. A prolonged inflammatory response with chronic microglia activation is associated with deleterious effects on cognitive behavior.

Stem cell therapy in preclinical experiments restores the BBB. Repair of the BBB was demonstrated using a CCI injury model in rodents with either human MAPC or autologous cells from the tibia of the animal. Administration of multiple doses (MAPC) or a single dose (autologous cells) resulted in a decrease in BBB permeability after TBI as

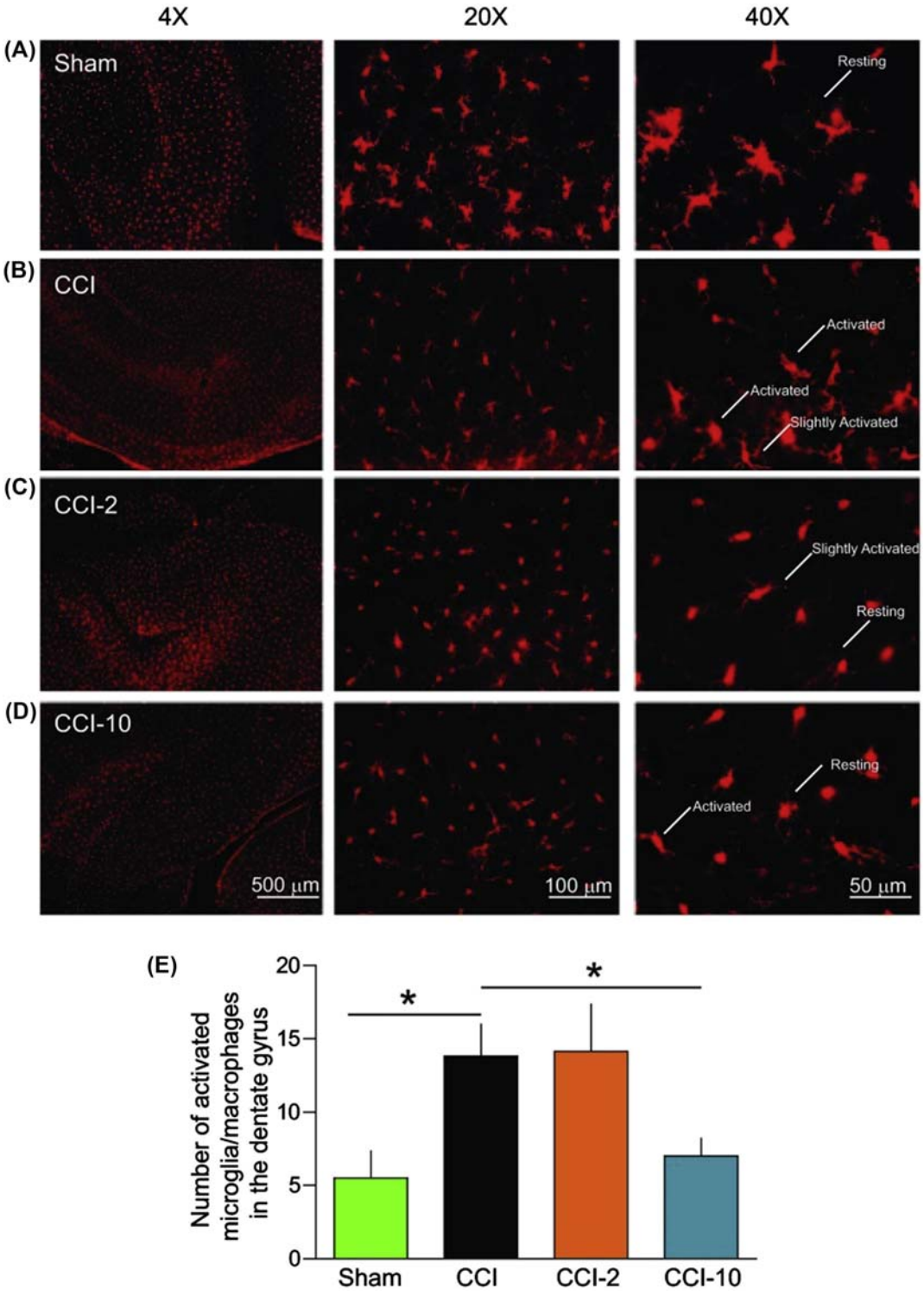

FIGURE 23.4 **Multipotent adult progenitor cell treatment reduced the number of activated microglia in the dentate gyrus.** (A–D) Photomicrographs of microglia from sham (A), CCI (B), CCI-2 (C), and CCI-10 (D) animals using IBA1 antibody (*red*). Scale bars = 500 μm (magnification ×4), 100 μm (magnification ×20), and 50 μm (magnification ×40). (E) Number of activated microglia in the dentate gyrus of the ipsilateral hippocampus. *$P \leq .05$. *CCI*, cortical contusion injury (untreated); *CCI-2*, cortical contusion injury treated with 2 million cells/kg; *CCI-10*, cortical contusion injury treated with 10 million cells/kg.

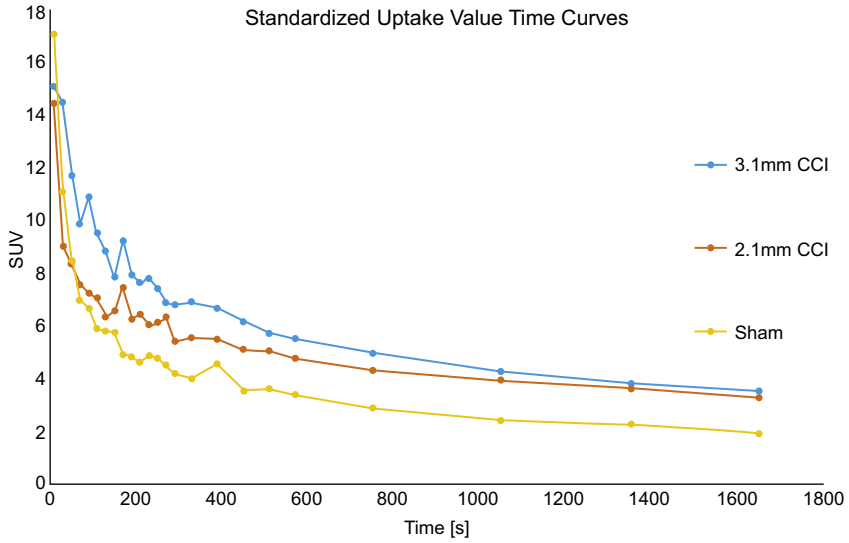

FIGURE 23.5 Whole-brain standardized uptake values (SUV) relative to muscle of the radioligand [^{11}C]PBR28 after a traumatic brain injury (TBI) in a rodent model. There was a dose-dependent increase in the amount of [^{11}C]PBR28 uptake depending on the severity of the injury. *2.1 mm CCI*, 2.1-mm controlled cortical impact simulating a moderate TBI; *3.1 mm CCI*, 3.1-mm controlled cortical impact simulating a severe TBI.

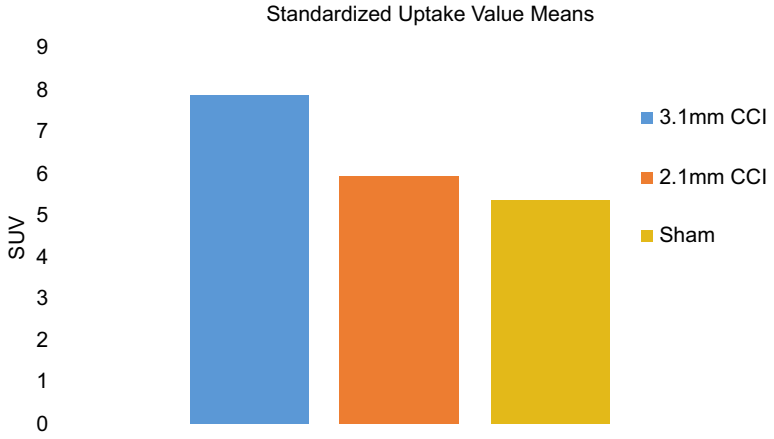

FIGURE 23.6 Microglial counts based on morphology in the thalami after a traumatic brain injury (TBI) in a rodent model. Severely injured animals had the highest counts of all cellular morphology types, all of which were statistically significant at $P < .05$. Microglia were identified via immunohistochemistry using the antibody Iba-1. Manual counts based on specific morphologic criteria were then carried out. *2.1 mm CCI*, 2.1-mm controlled cortical impact simulating a moderate TBI; *3.1 mm CCI*, 3.1-mm controlled cortical impact simulating a severe TBI; *SUV*, standardized uptake values.

measured by extravasation of Evans blue dye [30,31]. BBB integrity was further examined by immunostaining for the tight junction protein, occludin. A qualitative increase in occludin staining occurred in CCI-injured animals that received both forms of cell therapy, compared with the CCI-injured animals alone. Pati et al. were able to clearly elucidate a molecular mechanism for the effects of MSC on the BBB in TBI. They found that a soluble factor produced by MSC, tissue inhibitor of matrix metalloproteinase-3 (TIMP3), inhibits vascular endothelial growth factor A—induced breakdown of endothelial adherens junctions. Increased circulating levels of TIMP3 led to the replacement of endothelial function and BBB compromise. In addition, they discovered that the attenuation of TIMP3 expression in MSC negates the effects of MSC on BBB permeability, whereas intravenous (IV) administration of recombinant TIMP3 alone inhibits BBB permeability [32].

Cerebral Edema

Cerebral edema results in reduced cerebral microvascular perfusion, leading to significant secondary neuronal injury after TBI. Cytotoxic and vasogenic cerebral edema lead to increased brain tissue water and a subsequent

rise in ICP [33]. Cytotoxic edema is caused by the establishment of an osmotic gradient causing water flow into the intracellular space. The osmotic gradient is established when there is an influx of intracellular ions from active ion pump failure and increased membrane permeability. Vasogenic edema is also caused by an osmotic and oncotic gradient; however, the ion and protein flux is from the intravascular to extracellular space. Vasogenic edema follows disruption of the cerebral vascular endothelial cell lining that makes up the BBB [34]. The consequential elevations in ICP serve as a clinical target and surrogate marker of cerebral edema. Restoration of the BBB with stem cell therapy allows better treatment of cerebral edema; clinical trials have shown a reduction in therapeutic intensity required to counter cerebral edema in brain-injured patients treated with bone marrow mononuclear cells (BMMNCs) [35].

CURRENT TRAUMATIC BRAIN INJURY MANAGEMENT STRATEGIES

Current TBI management strategies are designed to minimize secondary injury, because the primary injury is considered irreversible [36–38]. The current treatment paradigm can be divided into surgical or medical management, depending on the severity of the injury. Surgical intervention focuses on evacuating extraaxial fluid collections and the placement of ICP monitors. Medical management of cerebral hemodynamics involves optimizing the CPP. The definition of CPP is the difference between the mean arterial pressure (MAP) and the ICP (CPP = MAP − ICP). Thus, maximization of CPP can be achieved by either increasing the MAP or reducing the ICP [39,40]. Variations in the precise techniques used to manage these variables do nothing to reverse the primary injury. Moreover, currently there are no effective treatments to prevent the secondary brain injury mechanisms after TBI. Therefore, significant advances in reducing the disability associated with TBI are unlikely using current management strategies alone [41].

PRECLINICAL DATA SUPPORTING STEM CELL THERAPIES FOR TRAUMATIC BRAIN INJURY

Cell Types

Two major classes of stem cell–based therapies have emerged for CNS insults: autologous and heterologous (allogeneic). The allogeneic class can be broken down into embryonic, embryonic-like (induced pluripotent stem cells cells), and adult cells. Adult stem cells are derived from a niche in a developing or developed organism [42]. These cells are considered multipotent or pluripotent because they can differentiate into multiple adult cell types. The adult subdivisions includes fetal, specialized niche cells (for example, subventricular zone derived neural stem cells [NSCs]), bone marrow–derived adherent cells (mesenchymal stromal cells [MSC] and MAPC), and nonadherent cells (BMMNCs and human umbilical cord blood cells [hUCBCs]). Autologous cells include cells used in heterologous applications, including BMMNC, expanded MSC, harvested niche cells such as NSC, and privately banked hUCBC.

Mechanisms of Action

The proposed biologic mechanisms for using cell-based therapy for TBI fall into one of two major categories: neural/supporting element replacement or an antiinflammatory/immune biologic response modifier. Early preclinical work focused on neural replacement mechanisms. The term “transdifferentiation” was popularized after studies showed that in vitro “adult” or nonembryonic progenitor cell types could be coaxed into neural phenotypes and express neural protein [43–45]. In vivo murine models replicated this intuitive hypothesis that progenitor cells migrated to an area of injury, transdifferentiated into neurons, and replaced neurons that were lost during the primary and secondary injuries [46–54]. Those reports showed impressive improvement in murine behavioral outcomes (improved locomotor recovery and temporospatial cognition) as well as apparent “engraftment” of these cells through 5-bromo-2′-deoxyuridine (BrdU) labeling identification. Immunohistochemistry sections of the injured brains demonstrated BrdU-labeled cells near the site of injury with neural phenotypes. However, this engraftment and transdifferentiation theory was later disproven and abandoned by most investigators. In 2006, Coyne et al. showed that the BrdU that was used to label MSC persisted in the area of injury, but it was merely the BrdU from dead MSC that persisted, not the cells themselves. Specifically, the thymidine analogues were taken up by other cells in the area of injury (endogenous NSC or microglia) and gave a false signal [55]. However, discarding the engraftment hypothesis did not change the improvement shown in behavioral outcomes in murine models.

Although some investigators still work with the hypothesis that progenitor cells transdifferentiate into neurons, most have turned to investigating progenitor cells as systemic neuroinflammatory modulators. The overall neuroinflammatory modulation of stem cells in TBI is downregulation of the intrinsic proinflammatory responses to injury and upregulation of antiinflammatory responses. Through probable pleiotropic mechanisms, systemic administration of cellular therapies improves outcomes compared with placebo. In this way, progenitor cell therapy preserves neurons that have not yet died from the primary injury but are vulnerable as a result of ongoing secondary injury [31,55–60]. Potential mechanistic pathways for this include downregulation of the activated microglia population to downregulate phagocytosis/neuron programmed cell death [31,60] and the study of antiinflammatory proteins secreted by progenitor cells such as TNF α–stimulated gene 6 product (TSG-6), Wingless-type MMTV Integration Site Family, Member 3a, and TIMP3 [61–64]. The success that was seen with TIMP3 and BBB preservation led Pati et al. to investigate this MSC-derived soluble factor for a neuroprotective role. They found that rodents treated with IV TIMP3 did not experience the significant loss of NSCs that is routinely seen in the preclinical literature [62]. NSC upregulation is important because NSC develop into immature neurons that migrate into the granule cell layer and integrate into neural circuitry. Others have produced some evidence of stem cell fusion with injured cells and the possible exchange of cytoplasm to preserve the damaged neurons. There is a growing body of literature that supports the concept that progenitor cell infusions profoundly upregulate multiple antiinflammatory neuroprotective pathways mediated by the reticuloendothelial system and possibly the lung [65,66]. Yang et al. demonstrated immunomodulation of the splenic response to stroke using human MAPC. MAPC treatment restored spleen mass reduction caused by stroke, elevated $T_{regulatory}$ cells within the spleen, increased IL-10, and decreased IL-1β released by splenocytes. In addition, MAPC treatment enhanced recovery in rats with intact spleens [67]. Prockop et al. revealed that IV infused human multipotent stromal cells (hMSC) become trapped as emboli in the lungs but upregulated a large increase in the antiinflammatory protein TSG-6. IV hMSC or TSG-6 administered during the initial mild phase of a mouse model of TBI decreased neutrophil extravasation, expression of matrix metalloproteinase-9 by endothelial cell and neutrophils, and subsequent BBB leakage. TSG-6 administered alone decreased TBI lesion size at 2 weeks and administration within 24 h showed improvements in memory, depressive-like behavior, and the number of newly born neurons 6–10 weeks after the injury [68].

Timing of Infusion

Stem cell delivery must take the acute injury time window into consideration. The secondary phase of brain injury occurs within hours of the initial trauma and is characterized by a rapid increase in proinflammatory cytokines at the site of injury and surrounding regions [13,69]. The peak in cerebral edema, and thus ICP elevations, follows the early inflammation after cytokine levels start to subside by 72 h. To downregulate the proinflammatory processes (or upregulate the antiinflammatory responses) associated with TBI, delivery of cell therapy likely must occur before or during the period of maximum inflammation. In clinical trials, this time point has been within 36–48 h of the trauma. The 36-h time window has been clinically reaffirmed in patients with stroke treated with MAPC. Patients treated before 36 h after a stroke had significant improvement relative to controls, whereas those treated after 36 h did not have the same benefit [70].

One potential error with this rationale is that the toxic cellular microenvironment may significantly affect the potential therapeutic benefit of the treatment. Cellular engraftment or even transient residence of the cells would cause most of the cells to die upon reaching the noxious region of interest. Harting et al. used immunohistochemistry to reveal that only 1.4%–1.9% of 400,000 infused NSC remained in the neural tissue at 48 h and 2 weeks after direct implantation after a CCI injury in rats [71]. This hypothesis led some investigators in the field to suggest that cell-based therapy be given 72 h after the brain injury to minimize the risk of transplanted cell death.

Two antiinflammatory mechanistic views have been hypothesized to explain the clinical benefits that are evident despite the seemingly uninhabitable injury area. First, stem cells of various types exhibit a paracrine secretion of growth factors and cytokines to salvage “at-risk” cells in the penumbral regions around the primary injury [72,73] Second, infusion of stem cells systemically alters the innate, cellular immune response to injury favorably such that tissue is preserved and functional outcomes are improved [65,74]. The exact window for cell therapy delivery remains ill-defined. Further understanding of cell therapy mechanisms of actions will define clinical timelines for maximum therapeutic benefits.

Conventional Cell Delivery Routes

A fundamental question of stem cell therapy for any disease process is how to administer the cells. Optimization of stem cell therapies for TBI requires a route of delivery without a significant effect on cell viability and function. Routine routes of delivery for animal models and human clinical trials include infusions IV, intraarterially, or intrathecally, and direct stereotactic implantation.

IV infusion of cells is minimally invasive; it provides widespread cellular distribution and has demonstrated promising preclinical data. The reported low levels of cell engraftment in the toxic injury site likely make the IV route more efficacious than stereotactic delivery or intrathecal infusion. Furthermore, TBI is not typically confined to one region of the brain, in contrast to a stroke that manifests symptoms in a defined arterial distribution. The disseminated injuries of TBI thus make direct stereotactic injection or targeted intraarterial infusion appear less effective than the widespread IV route of delivery. The Chopp laboratory exhibited both motor and cognitive improvements after the IV infusion of MSC 24 h after a CCI injury in rats. IV infusions of hUCBCs have exhibited enhanced neurogenesis [75], oligodendrocyte, and white matter (WM) protection [65], and decreased messenger RNA of proinflammatory cytokines and nuclear factor-κB (NF-κB) in rodents [76]. Similar to their work with MSC, the Chopp laboratory found significantly reduced motor and neurological deficits in brain-injured rats treated with IV hUCBC compared with control groups [47]. IV hUCBC therapy combined with granulocyte−colony-stimulating factor (G-CSF) demonstrated vigorous and long-lasting motor recovery compared with hUCBC therapy alone [77].

Pulmonary "First-Pass" Effect

IV-infused cells must first pass through the pulmonary vasculature, during which they are subjected to sequestration, the "pulmonary first-pass effect." Investigations in multiple laboratories have shown that IV-injected MSC (15−19 μm in diameter) undergo a significant pulmonary first-pass effect and become trapped in the pulmonary vasculature (5−7 μm in diameter) [78,79]. Fischer et al. found that less than 1% of injected MSC cross the pulmonary vasculature into the arterial circulation. As seen in Fig. 23.7, infrared imaging of the lungs, heart, kidney, spleen, and liver of rats immediately after IV infusion of varying cell types revealed accumulation of most MSC, MAPC, and NSC in the lungs [78]. Antiadhesion molecule strategies and rheologic agents (P188) did not improve the transpulmonary passage of these cells. In contrast, BMMNC (5−8 μm in diameter) pass through the pulmonary vasculature to the arterial circulation at a 30-fold higher rate [78]. A large volume of the infused BMMNC was noted in the spleen. The neurologic benefits that continue to be seen from IV MSC therapy, despite their significant pulmonary first-pass effect, challenge the idea of engraftment at the site of injury as a requirement for therapeutic efficacy.

Conventional Cell Delivery Routes, Continued

Intraarterial infusion of stem cells via the carotid artery is an attractive infusion method because of the focused delivery of cells and the potential for improved levels of cellular engraftment. Although it is invasive to access the carotid artery, consolidated injury areas close to the cerebral vasculature may see benefits from this style of delivery. Osanai et al. used in vivo optical imaging and immunohistochemistry to demonstrate significant engraftment of bone marrow stromal cells (BMSC) transplanted through the ipsilateral internal carotid artery. The authors also used near-infrared fluorescence imaging to demonstrate the transplanted BMSC migrating from the cerebral capillary bed to the injured CNS within 3 h of infusion [80]. Improved MSC engraftment rates are likely evident because these cells avoid the significant pulmonary first-pass effect that is seen with IV infusion. A major limitation of intraarterial infusion was observed when the large size of the injected MSC resulted in impedance of cerebral blood flow [81].

Intrathecal infusion via lumbar puncture allows stem cells to circulate rapidly in the cerebrospinal fluid (CSF); preclinical work has shown the potential for better engraftment rates by avoiding the pulmonary first-pass effect. Intrathecal delivery of stem cells has largely been used in preclinical spinal cord injury studies; however, the promising results invited TBI investigators to explore this route of delivery. Liu et al. found improved cortical engraftment and motor functioning when comparing MSC injected intrathecally versus IV in a rabbit model of TBI [82]. Walker et al. demonstrated enhanced neuroprotection when intrathecally transplanted MSC activated resident NSC NF-κB activity, which led to an increase in IL-6 production and a decrease in apoptosis [83]. Additional preclinical work on intrathecal delivery has been minimal. However, several clinical trials that used this mode of transport will be discussed in the clinical trials section subsequently.

Preclinical work that used direct stereotactic implantation of various stem cell lines displayed amplified engraftment and encouraging functional outcomes. However, this mode of delivery would require an invasive

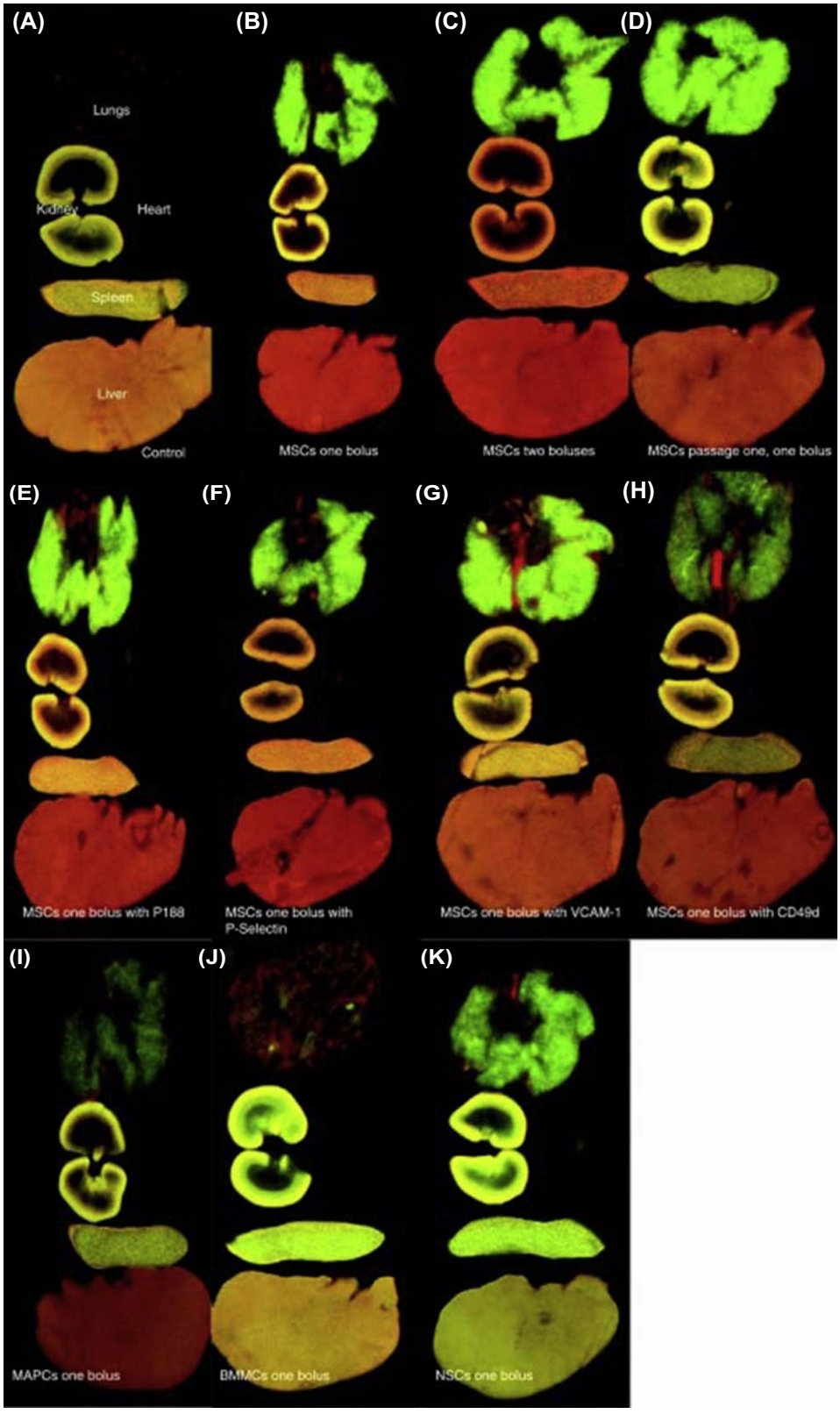

FIGURE 23.7 Infrared imaging of organs immediately after intravenous stem cell infusions. Lungs, heart, kidney, spleen, and liver of rats immediately after intravenous stem cell infusion (B–K) or vehicle (A, control) imaged ex vivo on the Li-COR Odyssey infrared imaging system. *BMMCs*, bone marrow mononuclear cells; *MAPCs*, multipotent adult progenitor cells; *MSCs*, mesenchymal stem cells; *NSCs*, neural stem cells; *VCAM*, vascular cell adhesion molecule.

neurosurgical procedure. Despite this constraint, rodent experiments displayed migration and engraftment of MSC at the injury site, increased endogenous cellular proliferation, and functional improvement up to 8 days after injury [51,84]. Several studies have been performed with direct implantation of NSC. As mentioned earlier, Harting et al. found that stereotactically placed NSC 1 week after a CCI injury in rats displayed a 1.4%−1.9% engraftment rate along the injection tracks 48 h and 2 weeks after placement. These animals had an observed improvement in motor function via the rotarod test; however, no improvement was found in cognitive function [71]. Li et al. examined and compared stereotactically placed NSC and NSC that were activated with *N*-acetyl aspartyl-glutamate synthetase (NAAGS/NSC). Both treatment groups displayed significant recovery of motor behavior compared with the control group. However, animals treated with NAAGS/NSC showed significantly improved motor behavior at 14 and 21 days compared with the NSC group ($P < .05$). In addition, NAAGS/NSC treatment inhibited cell apoptosis detected by terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labeling staining and the expression of proinflammatory cytokines (IL-1B, IL-6, and TNF-α) analyzed by enzyme-linked immunosorbent assay [85]. Wu et al. found that CCI-injured mice injected intracerebrally with primed human NSC showed significantly reduced injury-dependent accumulation of the axonal injury protein amyloid precursor protein. Intracerebral human NSC transplantation also reduced microglia activation and showed a transition toward the M2 antiinflammatory phenotype [56]. However, in addition to the invasive nature of this delivery route, another major barrier to direct implantation regards TBI as a diffuse injury with multiple large injury cavities. Multiple stereotactic injections would be needed in most instances to deliver cells to all injured areas of the brain. In addition to the risks associated with each procedure, the repetitive injections could exacerbate the underlying inflammatory response to injury.

Novel Cell Delivery Routes

Each of these conventional routes of cell delivery have major limitations; therefore, novel delivery methods are needed to enhance cellular migration, survival, and engraftment. Novel forms of delivery include nasal administration, scaffold constructs, hydrogels, and enhancement of stem cells to promote engraftment at the site of injury. Effective intranasal administration of stem cells has been reported in several neurologic disorders, stroke, neurodegenerative disorders, etc. [86]. The trigeminal nerve or cranial nerve V is a large sensory and motor nerve with many branches. Nerves providing sensation to the surface and interior structures of the face converge and enter the brain at the level of the pons. Intranasally administered cells follow the path of the trigeminal nerve and enter the brain parenchyma and CSF to carry out their functions on the CNS.

Success with transplantation of stem cells onto three-dimensional bioactive scaffolds in other injury paradigms have led to a large number of preclinical studies in TBI research. Natural materials such as collagen, hyaluronic acid, and porcine urinary bladder are the most commonly employed materials used for scaffolds. Wang et al. transplanted NSC cultured in a bioactive scaffold derived from porcine urinary bladder matrix (UBM) to a rat brain after a CCI injury. UBM has intrinsic antiinflammatory properties and it degrades rapidly in vivo, which makes it an ideal scaffold. Exposure to UBM alone decreased the loss of sensorimotor skills; however, only UBM containing NSC attenuated memory and cognitive impairments [87]. Guan et al. used PET imaging to show increased retention of human bone marrow MSC transplanted on collagen scaffolds in the lesion site of injured rats versus those transplanted without a scaffold. Brain metabolism was also improved in the collagen scaffold−implanted group as demonstrated by PET [88]. Synthetic materials are not as commonly employed in the use of scaffolds. However, Skop et al. were able to use a chitosan-based scaffold impregnated with growth factor (fibroblast growth factor-2) successfully to deliver fetal rat neural precursor cells to the injury site [89].

Hydrogels are three-dimensional networks of cross-linked hydrophilic polymers held together by covalent and hydrogen bonds that allow attachment, growth, and migration of surrounding cells onto the scaffold. Hydrogels have excellent nutrient and oxygen permeability allowing for stem cell survival [90]. The polymer networks expand when in contact with water; as such, they form in situ after injection. Hydrogels can be modified with proteins, glycosaminoglycans, cytokines, drugs, and other factors that will stimulate cell adhesion and growth [91]. Cheng et al. linked the laminin adhesion motif isoleucine-lysine-valine-alanine-valine (IKVAV) to a self-assembling peptide AcN-$RADA_{16}$ to form a hydrogel with mechanical stiffness similar to that of brain tissue. Rat neural precursor cells were encapsulated in the $RADA_{16}$-IKVAV hydrogel and transplanted into rats after a 2-mm biopsy punch neocortical injury. Immunohistochemistry revealed that the hydrogel enhanced survival of the transplanted cells compared with cells infused in saline [92]. The broad capabilities of hydrogels to retain water, provide structural support, modify cell behavior, and take on many different forms make them attractive scaffolds for use in cell therapy for TBI.

In their metaanalysis, Chang et al. evaluated preclinical TBI data to determine behavioral effect sizes for both modified and nonmodified stem cells [93]. "Modified cells" are those genetically programmed to overexpress certain proteins such as G-CSF or TSG-6, or those implanted onto biological scaffolds before being applied as treatment. The authors found an increased treatment effect with modified treatments over and above that found with naive progenitor cell treatment when analyzed in aggregate. This finding suggests that cells enhanced by gene modification or impregnated on scaffolds may survive longer in vivo or potentiate downstream mechanistic effects. The mechanisms by which modified cells produce their treatment effect is continually under investigation.

CLINICAL TRIALS

Phase 1 Bone Marrow Mononuclear Cell Pediatric Trial

Background

Autologous bone marrow mononuclear cell therapy for severe traumatic brain injury in children [94] was a phase I clinical trial (NCT00254722) in pediatric patients who experienced a severe closed-head TBI as defined by a GCS of 5–8. The purpose of the study was to determine whether autologous BMMNCs are a safe treatment for severe TBI in children. Each patient received 6×10^6 mononuclear cells per kilogram body weight within 48 h of the TBI. After the bone marrow harvest and infusion, infusion-related toxicity was determined by pediatric logistic organ dysfunction scores, hepatic enzymes, Murray lung injury scores, and renal function. No detectable infusion-related toxicities occurred during this phase 1 trial.

Rationale for Using Bone Marrow Mononuclear Cells

We have focused on the use of autologous BMMNC for several reasons: (1) There are no immune barrier considerations; (2) these cells are 5–8 μm in size (versus 13–19 μm for MSC), which prevents the significant pulmonary "first-pass" effect and makes IV delivery more feasible [78]; (3) there are no in vitro culture or scaling issues for autologous application; (4) they are readily available; (5) there are no issues with uncontrolled replication as with embryonic stem cells or fetal cells; (6) there are no ethically objectionable issues with the cell type; and (7) there are evolving data on the efficacy and putative mechanism of action.

Longitudinal Outcome Measures

Clinical outcome measures and the imaging date were used to evaluate the children's response to stem cell treatment at 1 and 6 months after the TBI. All functional and neurocognitive outcome scores showed significant increases at 1 to 6 months follow-up. Volumetric magnetic resonance imaging (MRI) data obtained at 1 and 6-month after the TBI revealed no loss of whole-brain intracranial volume, gray matter (GM), or WM, or increase in CSF space. MRI is being planned for this group of patients to evaluate chronic structural changes. In addition, PET imaging using the radioligand [^{11}C]PBR28 will evaluate chronic microglial activation.

Reduction in Therapeutic Intensity: Pediatric Intensity Level of Therapy Scores

A retrospective cohort study was completed after this phase 1 trial to study the intensity of treatment required to counter cerebral edema caused by neuroinflammatory responses to injury [35]. Investigators compared the degree of neurointensive care intervention in the 10 patients with severe TBI treated with BMMNC versus age-, severity-, and time-matched controls. In the pediatric population, the Pediatric Intensity Level of Therapy (PILOT) scoring system for treatment intensity directed toward ICP management was developed [95]. The PILOT score for each patient was calculated at 24-h intervals starting from the time of admission through 21 days. The group treated with BMMNC experienced a statistically significant reduction in PILOT scores beginning at 24 h after treatment through week 1 ($P < .05$). The treated group received autologous BMMNC infusion between 24 and 48 h from time of admission, which correlated to the time of divergence of the PILOT curves. In the first week, the control group treatment intensity remained elevated whereas the treated patients followed linear deescalation in treatment intensity after cell therapy. The treatment group continued to follow a linear decline in therapeutic intensity through 21 days. The control group required equal or escalated therapy and did not approach the treatment group scores until after 2 weeks postinjury. Fig. 23.8 illustrates these trends.

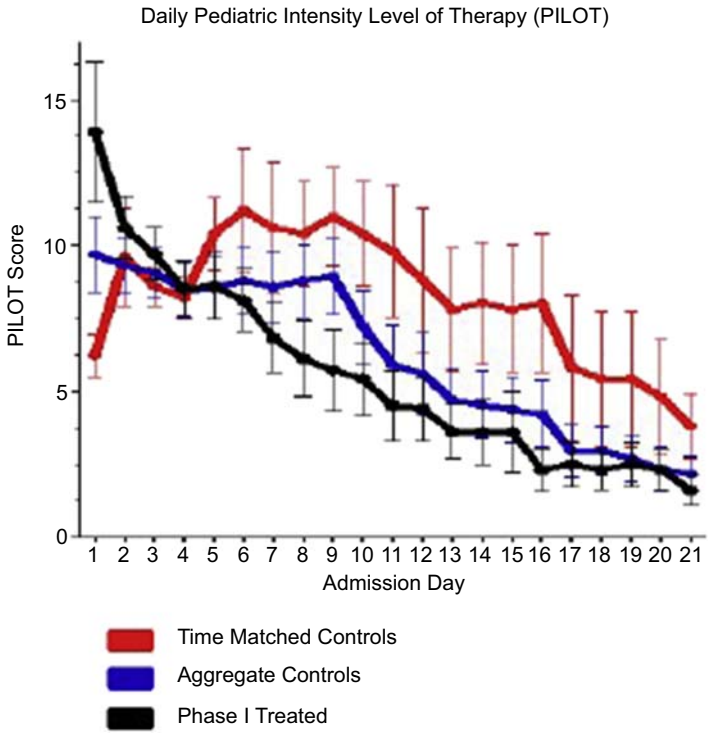

FIGURE 23.8 Pediatric Intensity Level of Therapy (PILOT) scores calculated from time of admission with divergence seen after time of cell therapy (patients were treated within 48 h of admission) ($P < .001$) compared with both 2006–2008 and 2000–2008 controls.

Conclusions

The pediatric phase 1 trial data demonstrate that autologous BMMNC infusion is safe, does not result in infusional toxicity, results in lower therapeutic intensity of neurocritical care, and preserves brain GM and WM. Although the study was not designed to study these parameters, outcomes suggested continued improvement in functional measures.

Phase 1/2a Bone Marrow Mononuclear Cell Adult Trial

Background

After completion of the phase 1 pediatric trial, a phase 1/2a study (NCT01575470) was carried out in adult patients aged 18 to 55 years who experienced a severe TBI [96]. In addition to safety and the logistic feasibility of autologous BMMNC for severe TBI, the investigators sought to determine a potential treatment effect size of WM structural preservation. Biomarkers of the inflammatory response to TBI were also evaluated sequentially before and after injury. A dose-escalation format was performed in 25 patients: five controls followed by five patients in each dosing cohort (6, 9, and 12×10^6 cells/kg body weight), and then five more controls. No serious adverse events were related to the study protocol.

Imaging Data

Imaging was performed at 1 and 6 months from the time of the TBI to acquire volumetric data and diffusion tensor imaging (DTI) comparisons. Significant differences and trends in structural preservation in global volumetrics of WM were seen in the treated versus untreated groups despite two confounding patients. These two patients had higher severity of injury and both required decompressive surgery before cell infusion, which altered the imaging readouts.

Prior imaging studies reported major impacts of TBI on the corpus callosum (CC) and corticospinal tract (CST), which led investigators to select these regions for the analysis of DTI metrics [97]. DTI data yielded measures of WM integrity, which included fractional anisotropy (FA) and mean diffusivity (MD). FA is a summary measure of microstructural integrity. Oversimplified, high FA values are a surrogate measure of coherent, tightly packed, and

myelinated fibers; higher FA is "good." The FA values of the CC in the untreated group were lower relative to the low-, middle-, and high-dose treatment groups at 1 and 6 months. The pooled treated patients exhibited higher FA values at 1 and 6 months relative to the untreated patients. MD is an inverse measure of cell membrane density and is sensitive to edema and necrosis; lower MD is "good." The MD values of the CC in the untreated group were higher relative to the low- and middle-dose treatment groups at 1 and 6 months. Pooled treated patients exhibited lower MD values of the CC at 1 and 6 months relative to the untreated patients.

The CST at the level of the brain stem exhibited similar characteristics of better WM integrity in the pooled group of treated patients as measured by DTI metrics (FA, MD, and radial diffusivity). FA values were higher and RD/MD values were lower in the pooled treatment group relative to the untreated group of patients.

Biomarker Analysis

Whole blood was collected in the acute and chronic periods for biomarker analysis. Cytokine levels of IL-1β, IL-4, IL-6, IL-10, interferon gamma (IFN-gamma), and TNF-α were assessed by enzyme-linked immunosorbent assay. The biomarker data showed a dose-dependent downregulation of the proinflammatory innate immune response to TBI, similar to the preclinical data in cell therapy models for TBI mentioned earlier. Investigators found a dose-dependent trend for TNF-α suppression and a statistically significant reduction in IL-1β, IL-10, and IFN-gamma in the high-dose group.

Conclusions

The adult phase 1/2a severe TBI trial found that autologous BMMNC infusion for adults with severe TBI is safe and logistically feasible. In addition, the investigators found a potential signal of treatment effect in terms of structural preservation of critical CNS architecture.

Phase 1 Mesenchymal Stromal Cell Subacute Traumatic Brain Injury Trial

Background

Autologous Bone Marrow Mesenchymal Stem Cell Therapy in the Subacute Stage of Traumatic Brain Injury by Lumbar Puncture was a phase 1 clinical trial (NCT02028104) in patients aged older than 6 months of age who were diagnosed with a chronic TBI of at least 1-month duration [98]. The study was a nonrandom, open-labeled, interventional cohort study. Patients with a persistent vegetative state (PVS) or disturbances in motor activity admitted to the Department of Neurosurgery at Yichang Center People's Hospital were selected for inclusion. A total of 97 patients (24 with a vegetative state and 73 with motor disturbances) voluntarily received bone marrow MSC via a lumbar puncture. Lumbar puncture was used as the delivery route in this study because the authors cited better cell engraftment rates, tissue sparing, and reduced host immune responses. No serious adverse events occurred upon MSC infusion. The primary outcome measure was change in the clinical symptoms of TBI within 6 months.

Rationale for Using Mesenchymal Stromal Cells

The authors highlighted MSC therapy as a novel method to treat TBI and discussed the potential mechanisms by which MSC improve functional disorders associated with TBI. Human bone marrow MSC have been the most widely studied progenitor cells for TBI therapy because of their ease of access and their multipotent differentiation potential. As discussed earlier, MSC accelerated neuroplasticity, facilitated neuronal regeneration, and improved functional recovery in preclinical data. Several mechanisms of potential therapeutic benefit are hypothesized, including the secretion of growth factors, exchange of genes and proteins through cell-to-cell fusion or contact, and induction of angiogenesis, and the effect on immunomodulation [99]. The authors acknowledged that engraftment and transdifferentiation were a controversial topic as the primary mechanism of MSC action. MSC were identified in this trial by the cell surface markers CD73, CD90, and CD105, which is the minimal criterion for characterizing MSC set forth by the International Society for Cellular Therapy.

Results

Despite long-term sequela (PVS or motor disturbances), 38 of 97 patients showed improvements 14 days after MSC therapy. The authors reported that 11 patients (out of 24 with PVS) showed posttherapeutic improvements in consciousness, which was evaluated according to the grading principle of PVS. The improvements seen were in responsive eyeball tracking, groaning, or tearing. Of 73 patients with motor disturbances, 27 showed improvements in motor function based on neurologic examinations.

Conclusions

The investigators concluded that MSC therapy through lumbar puncture is safe and effective in patients with severe TBI complications, such as PVS and motor disorders. In addition, the MSC effect was more pronounced in young patients and an inverse relationship was found between the time elapsed after injury and the outcome of cellular therapy.

Ongoing Clinical Trials

Phase 2 Multicenter Bone Marrow Mononuclear Cell Pediatric Trial

Pediatric Autologous Bone Marrow Mononuclear Cells for Severe Traumatic Brain Injury is a multicenter (Children's Memorial Hermann Hospital/UT Health and Phoenix Children's Hospital), prospective, randomized, placebo-controlled, blinded phase 2 safety/biological activity study (NCT01851083). A Bayesian adaptive dose escalation design is planned to assess doses of 6×10^6 and 10×10^6 cells per kg body weight versus placebo in children aged 5–17 years who are hospitalized for severe TBI (GCS 3–8).

The primary objective of the study is to determine the effect of autologous BMMNC on CNS WM and GM structural preservation. MRI-based quantitative indices of macroscopic (volume) and microscopic (FA and MD) integrity will be evaluated over time and compared with immediate postinjury measurements. Secondary objectives hypothesize autologous BMMNC infusion improving functional and neurocognitive deficits and reducing the neuroinflammatory response in children after TBI. Spleen size will be evaluated using ultrasound, because preclinical data have shown that the spleen responds to injury in the brain by releasing stored immune cells into the bloodstream. These cells then infiltrate the brain and promote a secondary inflammatory response that enhances neurodegeneration. Finally, infusion-related toxicity and long-term safety evaluations will be completed. Study enrollment commenced in August 2013 and is ongoing.

Phase 2b Bone Marrow Mononuclear Cell Adult Trial

Autologous Stem Cell Study for Adult TBI (Phase 2b) was designed in a fashion similar to that of the pediatric phase 2 trial (NCT02525432). It is a randomized, blinded, Bayesian continual reassessment method dose-escalation, placebo-controlled study design to treat severe, acute TBI in adults ages 18–55 years with an IV infusion of autologous BMMNC. The primary and secondary objectives between the two studies are the same. Enrolled patients are randomized to receive a single IV infusion of BMMNC (6×10^6 or 9×10^6 cells/kg body weight) or placebo. After cell infusion, safety data monitoring and MRI data acquisition have a timeline similar to that of the pediatric phase 2 BMMNC trial. Study enrollment commenced in November 2016 and is ongoing.

Phase 2 Modified SB623 Cells Trial

SanBio, Inc. is conducting a multicenter phase 2 trial entitled *A Double-Blind, Controlled Phase 2 Study of the Safety and Efficacy of Modified Stem Cells (SB623) in Patients With Chronic Motor Deficit From Traumatic Brain Injury* (NCT02416492). Patients aged 18 to 75 years with stable, chronic motor deficits resulting from focal TBI at least 12 months after the injury are eligible for enrollment. SB623 cells are adult bone marrow–derived cells that are transiently transfected with a plasmid construct encoding the intracellular domain of human Notch-1. These cells produce trophic factors that protect neurons in models of ischemic insult. In a rat contusion model of TBI, implantation of SB623 around the area of the injury resulted in significant improvement in motor function. A phase 1/2A dose-escalation study (NCT01287936) of SB623 cells stereotactically implanted into the brains of patients with chronic motor deficits caused by ischemic stroke showed statistically significant improvements in motor with no serious adverse events. The primary outcome is clinical efficacy of the cells as measured by changes in the Fugi–Meyer Motor Scale. In addition, the study will evaluate the safety and tolerability of the intracranial route of delivery. Enrollment opened in January 2016 and is ongoing.

Phase 1/2 Adipose-Derived Stem/Stromal Cells

Use of Adipose-Derived Cellular Stromal Vascular Fraction (AD-cSVF) Parenterally in Postconcussion Injuries and Traumatic Brain Injury is a randomized phase 1/2 trial for patients aged 16 to 70 years with concussion syndrome or mild TBI (NCT02959294). Patients must be 1 month postinjury and demonstrate repetitive TBI events (such as those that occur with contact sports), depression, cognitive disability, attention disorders, headaches, or other persistent changes after the TBI. Patients enrolled in the intervention arm will undergo microcannula harvest of autologous adipose-derived stem cells. Primary outcomes are concerned with safety, as measured by the number of adverse events, and changes in clinical symptoms associated with concussion TBI. Enrollment opened in November 2016 and is ongoing.

Phase 2 Mesenchymal Stromal Cell Neurologic Stem Cell Treatment Study (NCT02795052)

Neurologic Bone Marrow Derived Stem Cell Treat Study, or NEST Study, is a nonrandomized phase 2 trial for patients aged over 18 years with documented damage to the central or peripheral nervous systems. This study is not specific to TBI, but patients who have residual disability 6 months after the injury may be enrolled. Two intervention arms exist in this trial: (1) bone marrow MSC injected IV, and (2) bone marrow MSC injected IV and applied topically to the lower one-third of bilateral nasal passages. Primarily, the authors will be looking at activities of daily living, but they will secondarily examine deficits of neurologic function, such as speech, balance, hearing, and gait. Enrollment opened in June 2016 and is ongoing.

Phase 1 Collagen Scaffold–Mesenchymal Stromal Cell Study

The Safety and Efficacy Assessment of Injectable Collagen Scaffold Combined With Mesenchymal Stem Cells Transplantation in Patients With Brain Injury is a phase 1 trial for patients aged 35–75 years who are diagnosed with a severe TBI and CT evidence of intracerebral hemorrhage (NCT02767817). Patients who underwent craniotomy for hematoma evacuation are excluded because the sham group undergoes stereotactic hematoma evacuation. Treatment groups undergo stereotactic drainage of hematomas; however, one treatment arm then receives 10 million MSC via the drainage tube and the other treatment arm receives an injectable collagen scaffold with 10 million MSC. The primary outcome measure is the number of adverse events. Secondary outcome measures will survey functional outcomes (self-care and motor recovery), pain intensity, electrical brain activity, brain electrophysiology, and brain pathology diagnosed by CT scan. Enrollment opened in March 2016 and is ongoing.

CONCLUSION

There has been a growing body of preclinical literature supporting the use of various progenitor cell types to treat acute neurological injuries such as TBI and stroke. Preclinical work has laid the foundation for clinical trials, and although the exact mechanism of action is still not completely defined, it is complex and pleiotropic. There have been three metaanalyses that analyzed the efficacy of progenitor cell therapies for TBI in murine models [93,100,101]. Although each metaanalysis has limitations and includes studies with significant heterogeneity, all show statistically significant improvements in behavior outcomes. Jackson et al. also analyzed 14 studies that presented lesion volume metrics and the metaanalysis showed improvement in the size of lesion volumes after progenitor cell treatment. To date, this is the only histologic outcome metaanalysis in the preclinical TBI progenitor cell therapy literature. These findings from three independent metaanalysis teams are striking support that progenitor cell therapies are effective treatments for TBI. In addition, the authors of each metaanalysis performed multivariate regression to identify which cells were most beneficial, at which doses, and via which routes of administration (e.g., IV, intraarterial, direct injection in the lesion cavity). However, each metaanalysis found that there are not enough comparable data to make significant conclusions. This lack of conclusion is itself interesting: If there were large differences in effect size when different cells are administered different ways, we would expect that at least one of three metaanalyses would find it. This again suggests that a factor intrinsic to a progenitor cell administered in a systemic fashion has a role as the putative treatment mechanism.

Clinical trials in the acute, subacute, and chronic stages of TBI have highlighted the safety, logistical feasibility, and benefits of using stem cells as therapy for TBI. Ongoing clinical trials are seeking to demonstrate safety, CNS structural preservation, and improved functional outcomes. A variety of modified and nonmodified stem cells are being delivered via multiple routes and doses. The future of stem cell therapy for TBI will likely include genetically modified cells and repeated dosing regimens. Further preclinical understandings of cell therapy mechanisms of action will guide future clinical trial design.

As cell therapy becomes a more widely available treatment for TBI, it will become increasingly important to monitor these patients chronically. Equally important to administering cell therapeutics for TBI is the long-term ability to observe their clinical benefits. Patients enrolled in the pediatric phase 1 trial (NCT00254722) will undergo PET/CT imaging with the radioligand [^{11}C]PBR28 to identify chronic microglial activation years after the primary injury. These data will also highlight the role that stem cells have in the chronic neuroinflammatory response to TBI and will be correlated with long-term functional outcomes. Research efforts dedicated to imaging and laboratory techniques that can be used to observe response to cell therapy both in the acute and chronic stages of TBI will guide future preclinical works.

Continuing to find treatments that improve clinical outcomes after TBI is vital to decreasing the global burden of TBI, and we believe that progenitor cell therapy will have a fundamental role in these efforts.

List of Acronyms and Abbreviations

AIS Acute Injury Score
ALT Alanine Aminotransferase
ARDS Adult Respiratory Distress Syndrome
AST Aspartate Aminotransferase
ATLS Advanced Trauma Life Support
BBB Blood−Brain Barrier
BM Bone Marrow
BMMNCs Bone Marrow Mononuclear Cells
CC Corpus Callosum
CCI Controlled Cortical Impact
CNS Central Nervous System
CPP Cerebral Perfusion Pressure
CSF Cerebrospinal Fluid
CST Corticospinal Tract
CT Computed Tomography
DTI Diffusion Tensor Imaging
ESCs Embryonic Stem Cells
FA Fractional Anisotropy
GCS Glasgow Coma Score
GM Gray Matter
GOS Glasgow Outcome Score (EC-expanded, children)
hCG Human Chorionic Gonadotropin
hUCBC Human Umbilical Cord Blood Cells
ICP Intracranial Pressure
ICU Intensive Care Unit
ICV Intracranial Volume
IL Interleukin
iPS Cells Induced Pluripotent Stem Cells
ISS Injury Severity Score
IV Intravenous
LAR Legally Authorized Representative
LOD Logistic Organ Dysfunction
MAP Mean Arterial Pressure
MAPC Multipotent Adult Progenitor Cell
MD Mean Diffusivity
MHC Major Histocompatibility Complex
MHH Memorial Hermann Hospital
MNCs Mononuclear Cells
MRI Magnetic Resonance Imaging
MSC Mesenchymal Stromal Cell
NIH National Institutes of Health
NSCs Neural Stem Cells
PELOD Pediatric Logistic Organ Dysfunction
SOFA Sequential Organ Failure Assessment
TBI Traumatic Brain Injury
TNF Tumor Necrosis Factor
WM White Matter

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CHAPTER

24

Mechanical Determinants of Tissue Development

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INTRODUCTION

In biology and medicine, it has been common to study different diseases and pathologies from genetic and chemical factor perspectives that affect tissue development and function. In contrast, the effect of external mechanical forces has only more recently been documented for a significant role in cell signaling and tissue development and function [1,2]. Mechanical forces have been found to contribute significantly to the development and function of a variety of organs such as lung, heart, bone, cartilage, muscle, and brain, establishing a new field of science, called mechanobiology. Mechanobiology is an interdisciplinary field that combines developmental and cellular biology to understand the cellular response to mechanical stimulation and the mechanotransduction mechanisms by which these stimuli are translated into a cascade of biochemical events. Mechanical stimuli have an essential role in regulating cell functions such as survival, reproduction, and fate determination through changes in gene expression, protein synthesis, and assembly, which are crucial to maintain tissue homeostasis. Deviation from normal mechanical stimulation can affect cellular functions and, in turn, the composition and assembly of the extracellular matrix (ECM), which can lead to tissue and organ diseases. Examples of such malfunctions include fetal deformities, osteoporosis, osteoarthritis, tendinopathy, atherosclerosis, and fibrosis of different tissues [3–8]. An improved understanding of mechanotransduction mechanisms will unveil the pathogenesis of many developmental diseases to find treatments to cure or mitigate the consequences of these maladies. One way to explain mechanotransduction is to examine step-by-step mechanical signal propagation in the body from organs to single-cell nuclei in response to gravity or other mechanical loads applied to the body. First, skin receptors sense gravity or mechanical stimuli, which then transfer the signal to different types of tissues (e.g., bones, muscles, connective tissue, vascular tissue). Every tissue consists of different cells that are interconnected by an extensive network of ECM, including collagens, proteoglycans, and glycoproteins. This ECM network transfers the mechanical signals to the plasma membrane of cells, where signal transduction results in a cascade of biochemical reactions through cytoskeleton filaments toward the nucleus. The nucleus then regulates gene expression, resulting in the expression of new ECM components secreted from the cells as the external outcome of the input signals, finalizing the adaptation of the tissue to the mechanical stimulus.

The ECM senses changes in environmental forces and responds to these inputs by regulating gene expression inside the cell through signaling cascades from cell membranes to the nucleus. The nucleus transduces these signaling inputs to respond by secreting soluble and insoluble molecules as outputs from the cells as part of the cascade in mechanotransduction events. Thus, it is crucial to understand the major factors of the ECM, soluble molecules, and mechanisms of mechanotransduction, and they will be reviewed here.

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MECHANOTRANSDUCTION MECHANISMS AND MAJOR EFFECTORS

Cell Structure and Composition

The cells in the human body are routinely exposed to different types of external stimuli ranging from gentle touch to music and sensing gravity [8,9]. Mechanosensory transduction is the ability of living cells to sense a variety of mechanical stimuli and to convert these signals into electrical or biochemical intercellular signals [10–12]. Continuous responses to mechanical stimulation are possible owing to the evolutionary accumulated adaptations in cell structure and environment. All cells are surrounded by ECM and composed of complex plasma membranes with embedded ion channels and sensory receptors, and cytoplasm with organelles and a nucleus (except for red blood cells) [1,13,14]. The plasma membrane encloses the cells, defines their boundaries, and contains protein receptors that act as sensors of external signals. These receptors allow the cells to adapt continuously to changes in the extracellular environment [15]. Plasma membranes are composed of a 5-nm-thick phospholipid bilayer that serves as a relatively impermeable barrier to water-soluble molecules [16,17]. The membrane is composed of lipids containing hydrophilic, phosphorylated heads and hydrophobic tails, with embedded sensory receptors. In response to mechanical forces, the plasma membrane regulates cell attachment, cell–cell communication, and the motion of molecules across the lipid bilayer [18]. Moreover, the membrane has a key role in antigen recognition because of the high number of embedded receptors [5,14,19–22]. The cytoplasm contains cytoskeleton proteins and a variety of organelles such as mitochondria, Golgi apparatus, ribosomes, and the nucleus, which regulate cellular processes including mechanotransduction through the expression of genes (Fig. 24.1).

Extracellular Matrix

All cells reside in an ECM, which provides mechanical support to the cells through cell-attachment sites and functions as a reservoir of bioactive molecules that have a role in cellular functions. These matrix proteins interact with

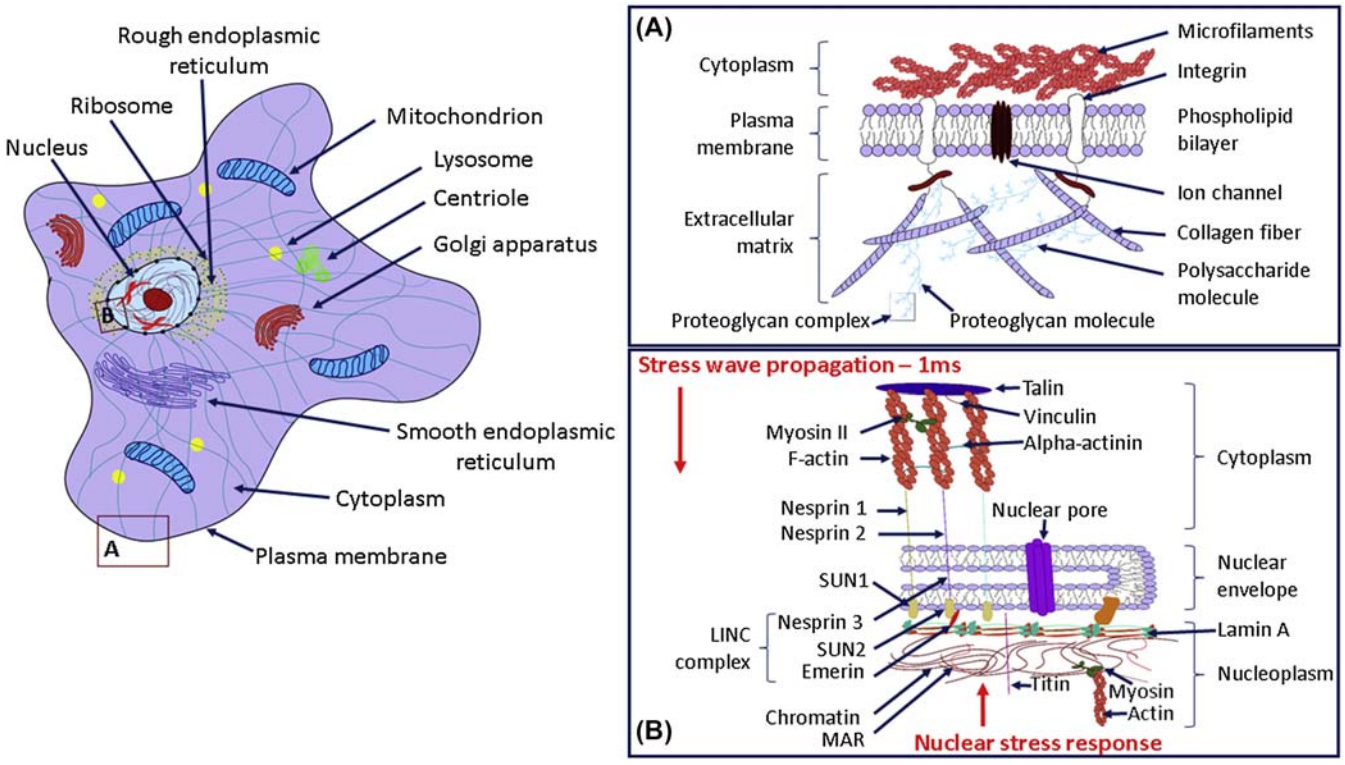

FIGURE 24.1 **Schematic of cell structure and the main mechanoeffectors.** (A) Various stimuli applied to the extracellular matrix (ECM) transmit the signal through integrins and other transmembrane receptors inside the cell cytoplasm, where microfilaments and microtubules transmit the inputs to the nuclear membrane. (B) The nuclear envelope is tightly connected with the cell cytoplasm and receives mechanical signals through nuclear pores and LINC complexes. After the nucleus receives the stimuli, there is a response through changes in gene expression, and subsequent regulation of the assembly of cytoskeletal and ECM components.

cells through cell surface receptors [14]. Collagens are a major component of the ECM in most tissues, about 28 different types of which have been characterized [23]. Molecules of collagen types I, II, and III constitute the bulk of the fibrillar collagens present across all tissues, particularly in dense connective tissues, and are arranged as insoluble fibrils. Such fibrillar structures resist compressive, shear, and tensile forces in tendons, bone, arteries, and skin. Collagen IV forms mesh-like scaffolds present as the basement membranes of vascular structures and in other tissues. The remaining dozens of collagens regulate fibril initiation, fibril diameter, and other factors in tissues; functions are still being discovered [14].

Proteoglycans are another important class of macromolecules in the ECM that form complexes with the protein structural components. Proteoglycans are macromolecules that consist of a core protein covalently bound to glycosaminoglycan (GAG) [24] chains. These molecules vary in function depending on the core protein, the GAG chains, and the sulfation patterns [25]. Aggrecan, for example, provides compressive stiffness in tissues. GAGs, which include chondroitin sulfate, dermatan sulfate, and keratan sulfate, are the major functional components of proteoglycans. Hyaluronan, a nonsulfated GAG, is hydrophilic and imparts enhanced lubrication in tissues such as cartilage. Many cell surface receptors for hormones and growth factors are also proteoglycans. Thus, proteoglycans fall into two categories: membrane-associated and secreted forms.

Glycoproteins such as fibronectin, laminin, vitronectin, and tenascin are found in the ECM and further mediate cell−cell and cell−ECM interactions by binding to collagens, proteoglycans, and cell surfaces. The expression of tenascins, found mostly in the nervous system, heart, smooth and skeletal musculature, tumors, and healing wounds, is mechanically regulated, which is turn controls cell adhesion and spreading [26,27]. The elastic recoil capabilities of various tissues such as dermal, pulmonary, and vascular are mainly controlled by elastin and fibrillin as insoluble elastic fibers [28].

All of the components of the ECM are uniquely organized in different tissues within a steady yet dynamic state. There is reciprocal cross-talk between the ECM and the resident cells, such that the cells with their mechanosensing capabilities offered through integrins and the cytoskeleton maintain the mechanical properties of the ECM; in turn, ECM deposition, rearrangement, or degradation is a critical regulator of cell and tissue health [14].

Ion Channels and Mechanoreceptors

Evolutionarily, the human body has accumulated a variety of cellular and molecular structures that can sense and respond to applied external forces; these includes but are not limited to mechanosensitive (MS) ion channels, G protein−coupled receptors (GPCR), integrins, and tight junction (TJ) proteins [29−31]. MS ion channels, among others, are believed to be the oldest and the most essential molecular force transducers owing to their ability to operate on a millisecond time scale, passing millions of ions per second through membranes [29]. MS ion channels change their confirmation in response to external forces such as pressure, gravity, shear stress, and osmolarity [31,32]. The existence of ion channels gated by mechanical stimuli was first suggested in 1950, when the phenomenon was described in association with the electrophysiological response of sensory neurons when stretching frog muscle [33]. Later, this phenomenon was observed with hair cells [34], skeletal muscle cells [35,36], red blood cells [37], cardiomyocytes [29,31,35,38], osteoblasts [39], skin cells [40], neurons [29,31,41], and many others cells.

The activity of MS ion channels is observed in all vital body functions, such as the maintenance of the synaptic cleft; synapse formation [5,41]; regulation of heart rate [42]; arrhythmia [3,38]; regulation of cell volume [30]; tactile [32,43], auditory, and visual sensations [44,45]; pulmonary arterial hypertension [46]; muscular dystrophy [36]; polycystic kidney disease [6]; and tumor progression [47]. The diversity of MS ion channels in prokaryotic and eukaryotic cells potentially involved in mechanosensory transduction has been summarized in prior studies [3−42]. Here, the attention is on the major eukaryotic MS ion channels and their function in health and disease.

Degenerin (DEG) and epithelial sodium channel (ENaC) proteins belong to a family of eukaryotic amiloride-sensitive, Na^+-selective ion channels that are touch-sensitive receptors [48]. Mutations in DEG genes in *Caenorhabditis elegans* triggered confined degeneration of touch-receptor neurons [49]. ENaC channels are expressed on the apical surfaces of diverse epithelial cells, including distal nephrons, gastrointestinal tract, skin, and cerebral arteries, where they regulate pressure-induced myogenic responses and are involved in maintaining tissue homeostasis and ion balances [50]. ENaC channels are active, have low voltage sensitivity, and can be blocked by some diuretics [51]. Studies have shown that ENaC mutants with upregulated expression display severe hypertension, whereas the opposite loss of function mutants demonstrate hypotension [52,53].

The transient receptor potential (TRP) channels received their name after they were first discovered in the *trp* mutant strain of fruit fly *Drosophila*, with its main feature of transient photoreceptor activity in response to light

[54]. In humans, there are 27 members of this family and they are encoded by 33 genes. There are six major groups of TRPs: TRPC (canonical), TRPV (vanilloid), TRPA (ankyrin), TRPM (melastatin), TRPP (polycystin), and TRPML (mucolipin) [29,31]. Here, we focus only on TRP receptors that are widely expressed in different tissues and perform critical functions. TRP channels are permeable to cations and are activated by a variety of physical and chemical stimuli; they have a crucial role in sensory regulation, calcium signaling, and the maintenance of homeostasis. TRPA1 (subfamily 1, member 1) receptor was described as a mechanical stress sensor and is highly expressed in nociceptive neurons [55], mechanosensory epithelia of the inner ear [35,56], pancreatic β cells [57], and periodontal ligament cells [58]. TRPC1 (subfamily C, member 1) receptors are widely expressed in brain cells, spinal neurons, cardiomyocytes, smooth muscle cells, endothelia cells, and others [21,29,31]. Another highly expressed receptor is TRPC6 in dorsal root ganglia neurons, cardiomyocytes, and variety of epithelial cells in the colon, esophagus, and stomach [29,59]. These TRPC6 receptors respond to most mechanical stimuli, tension, or osmotic forces. The function of these channels was controversial; they were activated upon sensing mechanical stimuli; however, they can also assemble into more complex heteromeric structures and mediate the activity of GPCR. Thus, they appear to perform different functions. Other members of the TRP family of ion channels perform functions ranging from thermosensation to sensing shear stress and pressure. For instance, TRPV4 channels are activated when physical forces are applied to integrins, allowing Ca^{2+} ions to flow into cells, which in turn leads to the activation of phosphatidylinositol-3-OH kinase (PI3K) and Rho [60,61], both of which are crucial molecular pathways that regulate cellular response to mechanical stimuli.

Piezo receptors were more recently discovered and have only two members, Piezo 1 and Piezo 2. They have a critical role in the function of hollow organs, such as the stomach, bladder, lung, and blood vessels, where they trigger transduction mechanisms by sensing shear stress [62–64]. Piezo 2 channels also have a critical role in proprioception; Piezo 2 knockout mice presented uncoordinated movement [5,43,62].

N-methyl-D-aspartate (NMDA) receptors are oligomeric cation channels that are present in the central and the peripheral nervous system, where they regulate essential physiological and pathological processes such as long-term potentiation, synaptic plasticity, and neurodegeneration via Ca^{2+} signaling [29,31,65–67]. These receptors are gated by mechanical forces and respond to changes in the osmotic and hydrostatic pressure. In addition, glutamate and glycine ligands are crucial for activation of these channels [65–67].

Besides ion channels gated by mechanical forces, some voltage-gated cation channels are also considered MS and activated in response to mechanical stimuli; however, they act through different activation mechanisms [29,31]. For instance, voltage-gated sodium channels $Na_v1.5$ or voltage-gated calcium channels $Ca_v1.2$ are present in electromechanical tissues such as muscle, heart, and gut [5,68,69]. Another large group of channels is potassium channels, $K_{2P,}$ with a unique two-pore structure; they are present in excitable and nonexcitable cells [70]. These channels regulate potassium flow conductance that is crucial to establish membrane resting potential, cell excitability, and transport of water and solutes to support ionic balance.

Cytoskeleton

The ECM is a complex structure of proteins directly linked to the cell cytoskeleton via cellular receptors, integrins, and other transmembrane proteins that regulate cell–cell attachment and cell–ECM interactions [71]. The cytoskeleton is a complex interconnected network of filaments that forms unique structures critical for the mechanical stability of the cells and for rapid responses to external stimuli. The cytoskeleton provides resistance to deformation; hence it maintains the mechanical stability of cellular structures (Fig. 24.2). The cytoskeleton is composed of three main components: semiflexible actin microfilaments [72], hollow microtubules (MTs), and stiff intermediate filaments (IFs). The actin cytoskeleton is composed of filamentous (F) actin, helical globular (G) actin, and a variety of actin-binding proteins that regulate the overall actin cytoskeleton structure [17,22,73]. The actin cytoskeleton is associated with both focal adhesion (FA) complexes at cell–ECM sites and with TJs at cell–cell sites (adhesion complexes attached to small binding proteins such as α-actinin, talin, or vinculin) [74]. Moreover, it has been shown actin MF can generate tension through the activity of molecular motors that convert adenosine triphosphate (ATP) into mechanical force. Actin filaments also serve a critical role in cell motion and migration through self-regulated ECM polymerization [1,17]. Alterations in actin assembly and structure triggered by mechanical forces regulate cell proliferation and gene expression [17,75]. A variety of cells (endothelial, osteoblasts, and mesenchymal stem) adhered to stiff substrates showed a shift toward higher F-actin versus G-actin ratios, which in turn triggered the activation of transcription factor Yes associated protein 1 (YAP1) [76,77]. YAP1 is a key effector of the Hippo molecular pathway, which is responsible for cell proliferation and differentiation [78]. This process is mediated

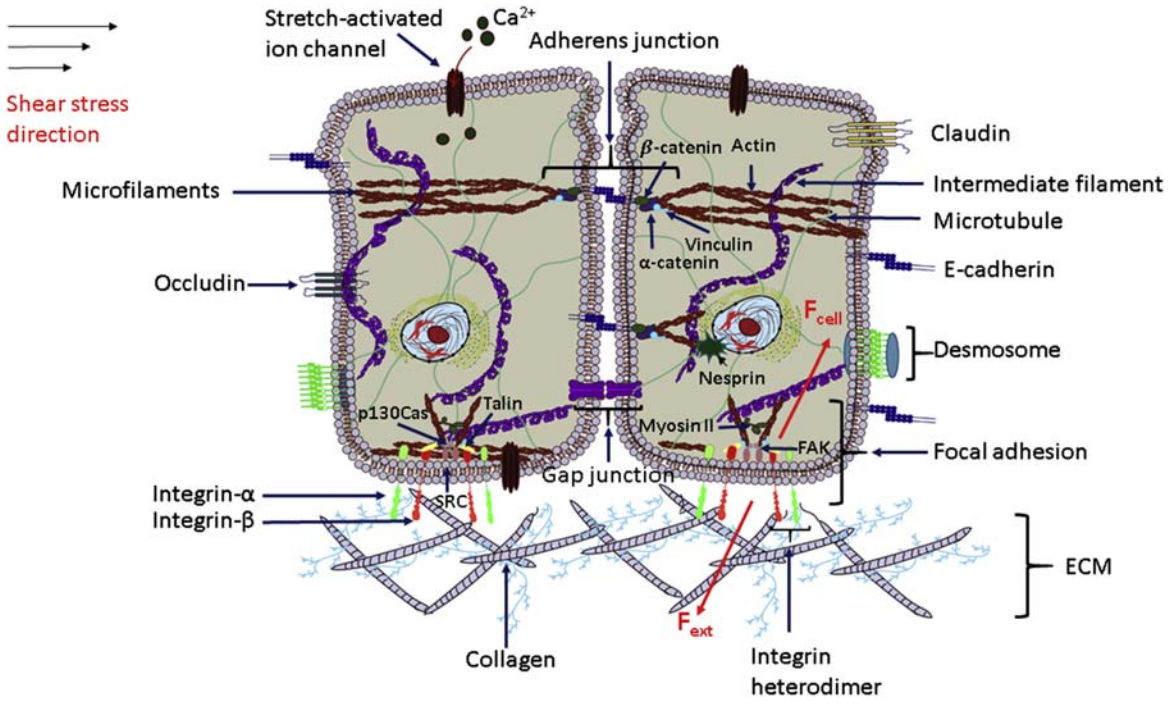

FIGURE 24.2 **Key players of cellular mechanotransduction.** Cell−cell connections transmit mechanical signals through adherent junctions, desmosomes, gap junctions, and tight junctions, which are ubiquitously present among cells. The signals between cells and the extracellular matrix (ECM) are transmitted through complexes of proteins called focal adhesions, which are mostly composed of integrins. The connections receive signals, perform sensory functions, and after microfilaments [72], microtubules [131] and intermediate filaments, they transmit the signal to the nucleus and then perform transmission functions. *FAK*, focal adhesion kinase.

by the formation of a complex with another transcription factor, transcriptional enhancer factor domain family member [79]. If these cells adhere to a soft surface, YAP1 coactivator becomes phosphorylated by serine/threonine-protein kinase, which in turn leads to YAP inactivation and, as a result, cell death [77]. Tubulin-based microtubules associated with the centrosome regulate the cell cycle. Microtubules are essential in cell mitotic spindle formation, vesicle transport, cell polarity, and cilia formation [80,81]. MTs have a major function in cell polarity and spindle orientation during the mitotic cycle, with associated regulation of chromatin orientation. Moreover, cilia axoneme consists of MTs and are crucial in regulating sperm motility and the flow of mucus, cerebrospinal fluid, and other bodily fluids upon sensing mechanical forces. Finally, elastic IFs (lamin, vimentin, desmin, and keratin) link nuclei and cell attachment sites (desmosomes) [82]. Lamin proteins form the inner support structure for the nuclear net and have an essential role in mechanotransduction. Compared with actin filaments and microtubules, IFs are relatively stable structures. IFs are highly expressed in neuronal (especially axons) and epithelial and muscle cells, which are constantly undergoing high mechanical stresses [83,84].

The assembly of more complex cytoskeletal structures is possible when microfilaments, microtubules, and IFs are supplemented by other proteins such as actin-bound myosin chains, which combine to form stress fibers around the periphery of cells. Cytoskeleton components in combination form a complex network and fill the interior of the cell. These components are continuously remodeled, such that monomers can be rapidly added or removed.

Microfilaments and microtubules are essential to maintain cell shape and polarization; they support a vast range of fundamental cellular mechanisms such as vesicle transport, cytoplasmic organization, chromosome segregation, and cell migration. IFs complexed with other components are crucial for cell−cell interactions and attachment and are required for healthy tissue and organ function during development and adult life. Actin and myosin coat the interior side of the plasma membrane and perform contractile and supportive functions for the membrane. The external forces that are propagated through integrins of the plasma membrane can be transduced as chemical modifications of the cytoskeletal structure at information-receiving sites and later transferred along the network as cascades.

To understand the function of the cytoskeleton in mechanotransduction better, tensegrity has been suggested [22,85]. This model proposes compensatory stress−strain organization of cytoskeletal components, in which compression-bearing elements (microtubules and ECM adhesion sites) withstand the stretching of surrounding

elastic elements (microfilaments and IFs). This complex organization of cytoskeletal elements thus allows the rapid exchange of energy with the surroundings and immediate cellular responses to fluctuating microenvironmental and mechanical stimulation. Continuous movement of actin–myosin complexes, formation of new FA sites and ECM adhesions, as well as osmotic forces result in the accumulation of physiological prestress or isomeric tension in many components of the cytoskeleton, according to the tensegrity model. Thus, these cytoskeletal adaptations and the compensatory stress–strain organization and physiological isomeric tension could explain the ability of cells to transduce signals over long distances and to convert mechanical stimuli into the cascade of complex biomechanical reactions in the nucleus [85]. Cellular mechanics have been described by other models, such as the cortical network, open cell foam, and percolation. However, these models do not account for the contribution of the ECM and microtubules toward cellular mechanics [86].

NUCLEUS AS THE CENTRAL ORGANELLE IN REGULATING MECHANOTRANSDUCTION

The nucleus is the central administrative organelle of the cell. This organelle performs three crucial functions: the storage of hereditary information, the regulation of key cellular processes such as cell growth, metabolism, proliferation, and protein synthesis; and mechanotransduction [87]. The cell nucleus is composed of the nucleolus and chromatin–DNA complexes that are protected by the nuclear envelope (NE), which has a high number of nuclear pores [88]. The nucleus is supported by the endoplasmic reticulum covered with ribosomes that are responsible for rapid RNA synthesis [89]. Although the nuclear content seems to be isolated from the cytoplasm by the NE, these two compartments are tightly linked structurally and physically by two protein complexes, the nuclear pore complex and the Linker of the Nucleoskeleton and Cytoskeleton (LINC) [90]. Because of this tight connection with the cytoplasm, the nucleus senses physical forces applied to a cell in seconds and transduces these signals into a cascade of biochemical reactions to remodel and adapt the cell to changing environmental conditions. Nuclei respond within short periods by physical changes to lamina, relocation of nuclear markers, and the nuclear position itself. Over longer durations, nuclei change their stiffness, thus affecting the stiffness of cell cytoskeleton and the microenvironment of the cell [91]. All of these observations demonstrate the communication of all cell compartments in response to external stimulation and the critical role of nuclei not only for immediate responses but for long-term changes in cell structure.

These studies support the tensegrity model, in which proximal stimuli can affect distal cell structures owing to the connectivity among all cell compartments [10]. The nucleus is tightly associated with integrins such that the distortion of adhesive sites, followed by realignment of the NE structure, nuclear displacement, and stiffening are regulated by the underlying net of lamin A/C proteins. In response to integrin stimulation, plasma membrane ion channels transmit the signals of changing forces through the actin network to mitochondria bound to microtubules [7]. Mitochondria immediately respond by the significant release of reactive oxygen species and trigger activation of numerous signaling molecules such as nuclear factor κB and vascular cell-adhesion molecule 1 [92].

To understand these cascades better, the molecular organization of the nucleus is important, with a double-layered membrane or NE that connects chromatin-containing nucleoskeleton to the cytoskeleton. The inner and outer membranes of the NE are interconnected with pores that mediate this communication between compartments. The inner membrane of the NE is supported by nuclear lamina composed of lamin proteins (lamins A, B, and C) and other incorporated membrane proteins such as lamina-associated protein 2α, emerin, inner nuclear membrane protein Man1 domain–containing members [91]. The organization of the NE is similar to that of the cell plasma membrane, with a high number of ions channels and inner actin support. The nuclear lamina is a dynamic structure linked to the nuclear chromatin domains and is responsible for the structural organization of chromatin and gene expression. Moreover, earlier research confirmed the association of the lamina with the cytoskeleton, observed via electron microscopy; molecular studies based on nuclear migration have supported this conclusion [89,93]. The complex of two protein families, synaptic nuclear envelope protein/nesprin and SUN (**S**ad1p and UNC-84) colocalized within the NE linked the nuclear lamina and cytoskeleton and were accordingly named the LINC [90]. Disruption of the LINC complex or ablation of lamina A/C proteins caused the disruption of cytoskeleton actin organization, which presumably was required to be linked with nuclear pores for proper assembly and organization. Intriguingly, independent physical force responses of isolated nuclei has been shown in in vitro studies, which points to the critical role of the nucleus in cell responses to mechanical forces [94,95]. Similarities in the organization of the cell plasma membrane and NE suggest the presence of resembling signal amplification mechanisms, well described for the cell membrane through FA sites, but not proved for NE. It was speculated the nesprin family of

proteins performs this amplification function [94]. Nesprin proteins unfold under tension, exposing binding sites and triggering further nesprin dimerization and the recruitment of other factors to support LINC complex stability and rigidity. Mainly lamina A and C proteins are responsible for nuclear stiffness in response to applied stress on the nesprin 1 protein of the LINC complex [94].

Mutations in most of the nuclear LINC proteins, or the lamina complex, are related to a variety of human pathologies such as different laminopathies (congenital muscular dystrophy, mandibuloacral lipoatrophy, cerebral ataxia, dilated cardiomyopathy, and others) [7].

CELLULAR MECHANOTRANSDUCTION MECHANISMS

Internal mechanical forces sensed by cell surface intramembrane receptors (integrins) are responsible for binding cells to the surrounding ECM, and cadherins for cell–cell contact and communication [2] (Fig. 24.3). Mechanical forces applied to cell–cell contact and cell–ECM adhesions affect cellular homeostasis, including single cells as well as surrounding tissues and organs [96]. The complex cell structure allows the detection of alterations in mechanical forces applied from outside or inside the cell, and to converts these signals into a cascade of biochemical events to regulate cell responses known as mechanotransduction [97]. To understand how the body responds to mechanical stimuli, it is critical to study mechanotransduction mechanisms starting from the single-cell level to the more complex levels of tissues and organs, a hierarchy of responses [98]. Cells respond to external forces by regulating the expression of ECM and other downstream effectors inside the cell. The cascade of molecular mechanisms that regulate cell responses to mechanical forces remains under study; however, some of the events are understood and are presented here.

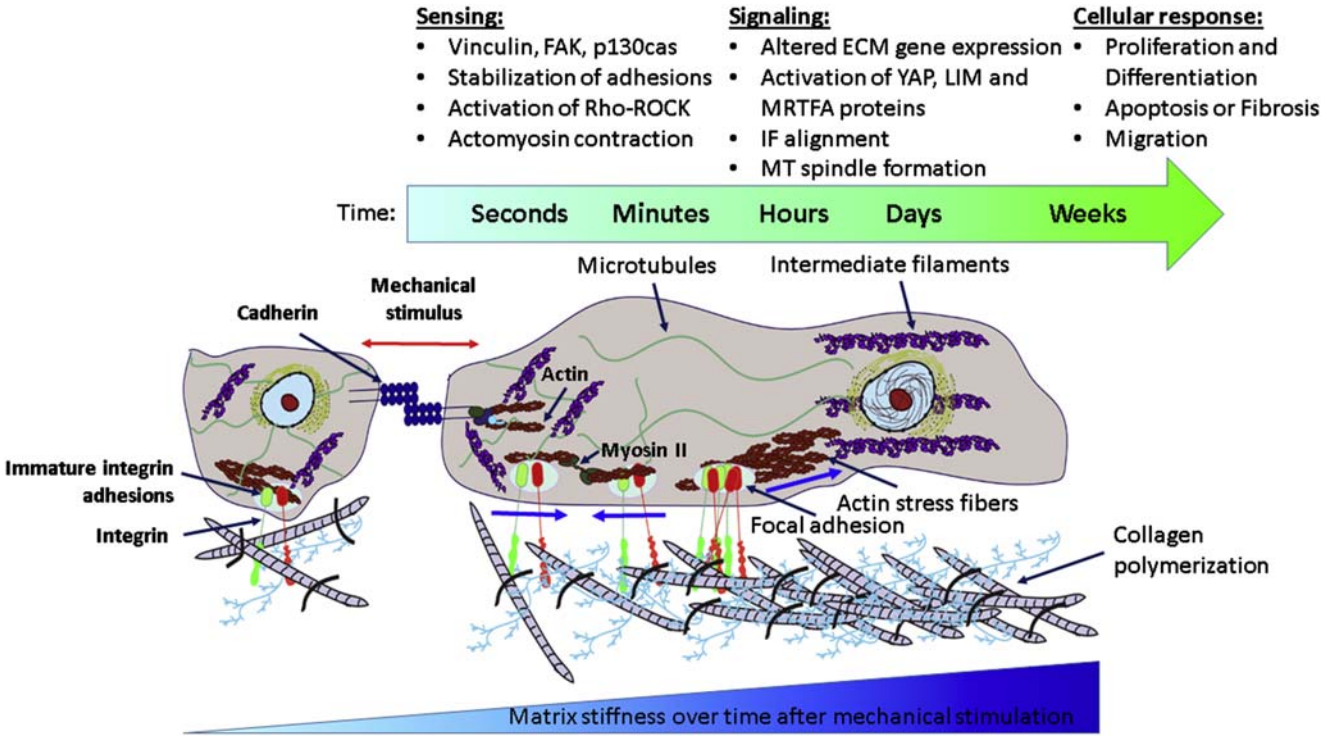

FIGURE 24.3 **Cellular changes over time after mechanical stimulation.** Cell–cell connections sense signals as stretching or shear stress, whereas cell–extracellular matrix (ECM) connections sense matrix rigidity and tension. The signals are converted into a cascade of biochemical reactions that cause cellular responses to the applied stimuli. Immediate changes after stimulation can include actin-myosin filament contraction, stabilization of focal adhesions, and the assembly of protein complexes. During the hours and days after initial signaling, the inputs reach the nucleus and cause changes by regulating major molecular pathways such as Yes associated protein (YAP) and LIM. The final chain of biochemical events can push the cell into division, differentiation, or trigger apoptosis or fibrosis, among other events, depending on the nature of the stimulation. *FAK*, focal adhesion kinase; *IF*, intermediate filament; *MRTFA*, myocardin-related transcription factor A; *MT*, microtubule; *ROCK*, Rho-associated protein kinase.

Mechanotransduction Through Cell—Extracellular Matrix Adhesions

All cells are surrounded by a network of ECM and are connected to this structure at multiple points through integrins of FA sites, to cytoplasmic actin filaments. Over 50 proteins are described in FAs, with integrins as the major component [17]. Integrins consist of two types of homodimers, α and β, which associate with the ECM and various intracellular proteins, such as FA kinase (FAK), talin, vinculin, paxillin, and p130Cas (also known as breast cancer antiestrogen resistance 1) [99]. The associated proteins maintain and regulate the activation of integrins, transduction of mechanical signals, linkage to the actin cytoskeleton, and additional functions. Upon cell attachment to the ECM, integrins bind and initially start to form small focal contacts consisting of integrins and other FA proteins, all of which form a complex FA [100]. Because of the essential role of FAs in regulating mechanotransduction signaling, they are considered to be mechanosensing centers from which the signals are oriented toward the proper organelle or structure to be transduced into a cascade of biochemical reactions [15]. These responses regulate cell adaption toward constantly changing environmental forces. FAs are stress sensitive and constantly alter their conformation under different stress conditions, to protect cells and support survival and stress resistance. These instantaneous changes in FA conformation are regulated by the Rho molecular pathway and its downstream effectors Rho-associated protein kinase (ROCK) and mDia (mammalian Diaphanous) [12,101]. The integrin linker protein talin is also sensitive to stress and undergoes unfolding and stretching in response to stress, revealing its binding site to vinculin to form the talin−vinculin complex. This complex triggers further changes in integrin structure and initiates signal transduction; it is also strongly dependent on myosin II activity. The talin−vinculin complex was shown to have a key role in the cell response to ECM rigidity [13].

FAK is a signaling molecule that has a critical role in regulating mechanotransduction by binding to various proteins such as receptor tyrosine kinase and PI3K, which are responsible for the activation and deactivation of molecular pathways [102,103]. Mitogen-activated protein kinase pathway (MAPK), with its major effectors such as extracellular regulated kinase 1/2 (ERK1/2), p38 MAPKs, and Jun N-terminal kinase (JNK), is upregulated upon FA complex formation [104]. The nucleus receives the signal after ERK1/2 and JNK activation, and via a feedback loop it regulates the expression of critical components of the ECM, such as collagen type I, which is responsible for tissue formation and remodeling [105].

Mechanotransduction Through Cell−Cell Adhesions

Adherent junctions (AJs) and desmosomes are the structures through which cells communicate with each other; and the have a role similar to that of FAs as mechanosensors, or mechanosensing ion channels and mechanotransmitters that include cytoskeleton components, nuclear lamina, and various transmembrane proteins [12,106]. AJs are composed of cadherins, Ca^{2+}-dependent transmembrane adhesion molecules, and intercellular proteins such as α-catenin, β-catenin, and vinculin [74]. Cadherin extracellular domains connect with cadherins of neighboring cells, whereas their cytoplasmic domain is responsible for the recruitment of catenins and other regulatory molecules. Cadherin−catenin complexes are critical in fetal development and in maintaining tissue homeostasis during adulthood [107]. Epithelial cells form a unique feature, TJs, on the apical side of AJs. These features are composed of specific membrane proteins such as occludin and claudin [108]. These TJs are crucial for barrier maintenance and the apical polarity of epithelial cells [109,110]. Several other pathways also regulate signal transduction though cell−cell contact. The Notch pathway regulates cell-fate determination in embryogenesis and tissue homeostasis in the adult state. Upon binding of Notch with its transmembrane ligand Delta, the extracellular domain is cleaved by ADAM (a disintegrin and metalloproteinase) protease, whereas the cytoplasmic domain is cleaved by γ-secretase [111]. The remaining Notch intracellular domain relocates to the nucleus, where it activates and deactivates targeted genes.

Cell−cell contacts share many functional similarities with cell−ECM contacts, such as clustering, recruitment of associated molecules for signal transduction, and trigger signaling cascades that result in activation of Rho family GTPases. Rho family small GTPases are important molecules that regulate cell cytoskeleton remodeling and reassembly in response to changing external forces. There are about 20 members in this family; three were described as regulators of cytoskeleton remodeling: RhoA, Ras-related C3 botulinum toxin substrate 1 (Rac1), and cell division cycle 42 [12]. RhoA protein is activated in response to mechanical stimuli, whereas its inhibition or its downstream effectors, ROCK or mDia, affect cyclic stress-induced cell filaments reorientation. Rac1 has a key role in regulating stress-induced responses in endothelial and other cells [17].

From Cells to Organs: How Mechanobiology Affects Tissue Development and Function

After understanding mechanotransduction mechanisms at the single-cell level, the next step is to understand how these mechanisms affect fetal development and overall tissue homeostasis. Mechanical forces are critical regulators of fertilization and fetal development from the earliest stages [11,12]. Cell motility during fetal development generates mechanical tension that shapes the embryo. Moreover, it significantly affects gene expression and thus cell differentiation [22,71,112]. Therefore, during fetal development there are two processes that play a key role, morphogenesis driven by tissue motility and cell differentiation controlled by gene expression [112].

Fertilization is regulated by mechanical forces, starting from sperm activity that is driven by the ATP-fueled motor protein dynein [12]. Dynein generates tail beats, which allow flagella to move the sperm within the viscous uterine environment toward an egg [113]. Upon sperm penetration inside the egg, a chain of biochemical reactions is triggered that allows the egg membrane to harden through cross-linking of molecular filaments [114].

The early-stage embryo is composed of tightly packed identical cell populations, until actin-driven asymmetrical division splits cells into polar and apolar groups [11,115]. The balance between symmetric and asymmetric cell division during fetal development is regulated by the mitotic spindle located inside the cell, which in turn is controlled by the physical forces generated upon interaction of cytoskeletal microtubules and actin microfilaments [116]. Correlations among cell geometry and the position and orientation of the spindle have been demonstrated [117]. The mechanism that regulates spindle position, and thus cell division, can be explained by the tensegrity model presented earlier [85]. Overall, at the early stages of embryonic development there is little to no ECM present, and thus cell differentiation and embryo development are driven by mechanical forces generated by cellular cytoskeleton proteins or by cell−cell interactions. One of the most well-described models is convergence extension during gastrulation [12,118−120]. Tissue narrowing and lengthening via cell intercalation generate mechanical tension along the embryo, driving morphogenesis. Elongation of the neuroectoderm is a passive process, with mechanical tension arising from the underlying chordamesoderm [121]. Only after the emergence of early epithelial cell layers does the ECM start to assemble, and at this point it is mainly composed of collagen type IV, laminin, and heparan sulfate proteoglycans accumulated at the base of trophectoderm [13,122]. After the ECM assembles, it contributes to the mechanical properties of the fetal microenvironment, and thus regulates cellular differentiation and the control of fetal development by controlling major molecular mechanisms that cause cell differentiation [123]. These features include the Hippo pathway, the conserved signaling pathway that regulates normal organ growth and development in humans and *Drosophila* [124−126].

When the main embryonic axis is formed, predifferentiated cells start to self-assemble into more complex hollow-like and other three-dimensional structures that become future organs. As in early embryonic development, morphogenesis is regulated by mechanical forces generated by cytoskeletal proteins and transmitted through ECM and cell−cell interactions to neighboring cells and tissues [127]. Another critical process contributing to normal tissue development and function is planar cell polarity (PCP) [118,120,128]. PCP regulates cell function in the perpendicular axis of the epithelial layer. Two major molecular mechanisms regulate PCP and are critical at the stage of embryonic morphogenesis: the core PCP and the Fat-Dachsous-Four-jointed modules [11,128]. The major role of these signaling pathways is to control the distribution of transmembrane receptors in opposing cell compartments of various tissues. This process in turn regulates cellular organization, motility, and function, based on the location of the signaling complexes. For instance, PCP regulates cell proliferation during wing development in *Drosophila*, as well as in the skin cracks of crocodiles [128].

Upon the establishment of a morphogenetic body plan, cells trigger differentiation into the organ-specific lineages and self-assemble into more complex structures that become organs [12]. At this stage of embryonic development, mechanical forces may not have a leading role, although they still contribute to organ development and overall body homeostasis. Soluble morphogens have a critical role at later stages of embryonic development, although some of these act by regulating cellular force homeostasis [129−131]. For example, during murine tooth formation, there is a sequence of events [12]. The process starts with increased production of growth factors responsible for cell migration and proliferation, which triggers massive cell migration toward each other, forming a defined condensed mesenchymal cell area [132,133]. The formation of dense tissue in turn triggers the activation of molecular pathways that regulate further tooth development. Finally, the dense mesenchyme initiates the production of collagen type VI containing ECM to harden the organ formed and finalize organ-specific lineage differentiation [11]. Thus, later embryogenesis is controlled by complex mechanochemical mechanisms that together control healthy organ development.

The development of some organs and body systems such as brain [4,134], heart [19,135], bone [136,137], lung [138,139], kidney [140], muscle [141,142], and the hematopoietic system [143−145] are affected by mechanical forces

more than other tissues and organs [20]. The early-stage sensitivity and responsiveness of embryonic stem cells to stretch and stress may still be present upon differentiation toward a defined cell type. For instance, fetal-derived cardiomyocyte progenitors maintain contractility crucial for healthy heart development on substrates with stiffness similar or equal to the native myocardial microenvironment [146,147]. Cytoskeleton formation and assembly of vimentin, filamin, and myosin were sensitive to substrate stiffness [147–149]. Loss-of-function experiments performed with ROCK inhibitors showed restored functions of cardiomyocytes on stiff substrates through the disassembly of stress fibers, which were shown to form in response to mechanical stress, such as stiff substrates [99]. Thus, phenotypic stage–dependent responses of single cells to mechanical stimuli at early stages of embryogenesis are important to the process.

Another example illustrates how the formation of lung buds from epithelial planar layers depends on force balancing in the developing embryo [22]. Cells located in more clustered areas of high turnover regions of cells in the epithelium demonstrate a greater chance of budding outgrowth; in comparison, the regions with higher ECM deposition remained the same size. Moreover, it was shown that budding could be modulated by cytoskeletal tension, causing a loss of differential basement membrane thickness [138,150]. The same budding pattern was observed in capillary sprout growth during angiogenesis [151]. Another example is kidney development, in which flow shear stress contributed to the assembly of glomerular structures, proper function for filtration and liquid flow, and morphogenetic control of nephron formation [152]. It was hypothesized that tissue-specific differentiation was strongly dependent on the properties of the substrate, such as stiffness, ECM composition, and elasticity, rather than mechanical stretching that is critical only during early stages of cell migration and orientation within the embryo.

Mechanical forces significantly affect embryonic development, but also tissue and organ function throughout adult life. Examples include the effect of physical exercise on the musculoskeletal system, blood flow stress on the cardiovascular system, cerebrospinal fluid and blood flow on brain function, and cyclic strain from inspiratory and expiratory forces on the pulmonary system [146]. Thus, normal tissue function and organ homeostasis strongly depend on proper mechanotransduction mechanisms between cell contacts and ECM in the tissue microenvironment. The human skeleton is one systems that can significantly remodel itself in response to physical forces. For instance, physically inactive patients experience osteopenia (bone mass loss) as a result of the lack of mechanical stress on bone and joints [12]. This process leads to increased bone fractures and joint malfunctions [153]. In contrast, an increase in the mechanical loading forces on bone tissue result in tissue hardening and matrix deposition [154]. The muscular system also modulates bone structure and composition via maintenance of tensegrity force homeostasis [155]. Similar to bone tissue remodeling, cardiomyocytes respond to hemodynamic changes in their microenvironment; under increased pressure, cardiomyocytes undergo hypertrophy, which in turn can lead to heart failure [156]. Numerous cardiovascular studies showed various factors contributing to both healthy and maladaptive maintenance of heart function, many of which are mechanosensors or mechanotransducers (stretch-sensitive ion channels, integrins and integrin-associated proteins, sarcomeric proteins, cell surface receptors, and others) [157]. These features support the importance of understanding the principles of mechanobiology. Strict control over tissue and microenvironment remodeling is crucial for organ homeostasis and to prevent devastating life-threatening diseases such as fibrosis and cancers associated with distorted remodeling of the ECM.

CONCLUSIONS

Mechanobiology principles are being applied in all biomedical fields. Mechanical forces have a crucial role in embryonic development and in the homeostasis and function of adult organs. Application of this knowledge in the field of tissue engineering and regenerative medicine will help in designing systems for the growth of artificial organs or tissues. Moreover, the insight and use of the principles of mechanobiology will allow the study of various diseases associated with changes in ECM assembly and misassembly and with cellular responses to mechanical stimuli.

Several microengineered platforms have been developed to recapitulate in vivo–like mechanical environment to decipher the role of mechanical forces in normal and pathological cellular processes [158]. Such platforms include bioreactors that apply tension and compression, microelectromechanical systems–based approaches that combine electrical and mechanical stimulation, and mechanopharmacology approaches that allow the simultaneous investigation of drugs and biomechanics in living systems [159]. However, the underlying mechanisms of how mechanical factors affect stem cell biology remain poorly defined. An understanding of the stem cell “mechano-niche” composed of cellular mechanical properties, the surrounding ECM, and external mechanical cues will enable the lineage-specific differentiation of stem cells for various in vitro and in vivo applications [160].

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CHAPTER

25

Morphogenesis, Bone Morphogenetic Proteins, and Regeneration of Bone and Articular Cartilage

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INTRODUCTION

Morphogenesis is the developmental cascade of pattern formation, establishment of body plan, and the architecture of mirror-image bilateral symmetry of musculoskeletal structures culminating in the adult form. Regenerative medicine is the emerging discipline of the science of the design and manufacture of spare parts for the human body including the skeleton to restore the function of lost parts owing to cancer, diseases and trauma. Regenerative medicine and surgery are based on rational principles of molecular developmental biology and morphogenesis and are further governed by principles of bioengineering and biomechanics. The three key elements for regenerative medicine and surgery are inductive morphogenetic signals, responding stem cells, and the extracellular matrix (ECM) scaffolding [1]. Advances in the molecular cell biology of morphogens will aid in the design principles and architecture for regenerative medicine and surgery. Regeneration recapitulates in part embryonic development and morphogenesis. Among many tissues in the human body, bone has considerable powers for regeneration and therefore is a prototype model for tissue engineering. On the other hand, cartilage is feeble in its prowess for regeneration (Fig. 25.1). Implantation of demineralized bone matrix into subcutaneous sites results in local bone induction. The sequential cascade of bone morphogenesis mimics sequential skeletal morphogenesis in limbs and permits the isolation of bone morphogens. Although it is traditional to study morphogenetic signals in embryos, bone morphogenetic proteins (BMPs), the primordial inductive signals for bone, were isolated from adult demineralized bone matrix. BMPs initiate, promote, and maintain chondrogenesis and osteogenesis and have actions beyond bone. The cartilage-derived morphogenetic proteins (CDMPs) are critical for cartilage and joint morphogenesis. The symbiosis of bone-inductive and conductive strategies is critical for regenerative medicine and is in turn governed by the context and biomechanics. The context in bone is the microenvironment, consisting of ECM scaffolding, and can be duplicated by biomimetic biomaterials such as collagens, hydroxyapatite, proteoglycans, and cell adhesion proteins including fibronectins and laminins. The rules

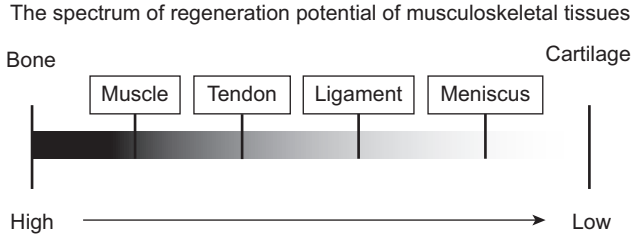

FIGURE 25.1 The spectrum of the regeneration potential of musculoskeletal tissues. Bone has the highest potential, and cartilage the lowest. Tissues with intermediate regenerative potential are muscle, tendons, and ligaments.

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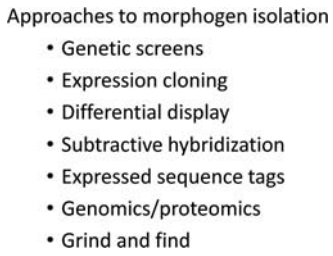

FIGURE 25.2 The various approaches to isolation of morphogens.

of architecture for regenerative medicine and surgery are an imitation and adaptation of the laws of developmental biology and morphogenesis, and thus may be universal for all tissues, including musculoskeletal tissues and a variety of other tissues in the human body.

The traditional approach to identifying and isolating morphogens is first to identify genes in fly and frog embryos by genetic approaches, differential displays, subtractive hybridization, and expression cloning (Fig. 25.2). This information is subsequently extended to mice and humans. An alternative approach is to isolate morphogens from bone with known regenerative potential. The principles gleaned from bone morphogenesis and BMPs can be extended to regeneration of bone and cartilage and other tissues.

BONE MORPHOGENETIC PROTEINS

Bone grafts have been used by orthopedic surgeons for nearly a century to aid in recalcitrant bone repair. Decalcified bone implants have been used to treat patients with osteomyelitis [2]. It was hypothesized that bone might contain a substance, osteogenin, that initiates bone growth [3]. Urist made the key discovery that when implanted intramuscularly, demineralized, lyophilized segments of rabbit bone induced new bone formation [4]. Bone induction is a sequential multistep cascade [5–7]. Important steps in this cascade are chemotaxis, mitosis, and differentiation. Chemotaxis is the directed migration of cells in response to a chemical gradient of signals released from the insoluble demineralized bone matrix. The demineralized bone matrix is predominantly composed of type I insoluble collagen and it binds plasma fibronectin [8]. Fibronectin has domains for binding to collagen, fibrin, and heparin. The responding mesenchymal cells attach to the collagenous matrix and proliferate, as indicated by [^{3}H]thymidine autoradiography and incorporation into acid-precipitable DNA on day 3 [9]. Chondroblast differentiation is evident on day 5, chondrocytes on days 7 and 8, and cartilage hypertrophy on day 9. There is concomitant vascular invasion on day 9 with osteoblast differentiation. On days 10–12, alkaline phosphatase is maximal. Osteocalcin, bone γ-carboxyglutamic acid containing Gla protein (BGP) increases on day 28. Hematopoietic marrow differentiates in the ossicle and is maximal by day 21. This entire sequential bone development cascade is reminiscent of bone and cartilage morphogenesis in the limb bud [7,10]. Hence, it has immense implications for the isolation of inductive signals initiating cartilage and bone morphogenesis. In fact, a systematic investigation was undertaken of the chemical components responsible for bone induction from the demineralized bone matrix.

This account of the demineralized bone matrix–induced bone morphogenesis in extraskeletal sites demonstrated the potential role of morphogens in the ECM. A systematic study of the isolation of putative morphogens from the bone matrix was initiated. A prerequisite for any quest for novel morphogens is to establish a battery of bioassays for new bone formation. The three key steps in bone morphogenesis are chemotaxis of progenitor stem cells, mitosis, and differentiation (Fig. 25.3). A panel of in vitro assays was established for chemotaxis, mitogenesis, and chondrogenesis, and an in vitro bioassay for bone formation. Although the in vitro assays are expedient, we routinely monitored a labor-intensive in vivo bioassay because it is the only bona fide bone induction assay.

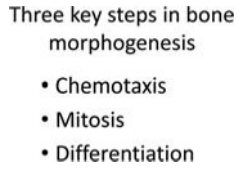

FIGURE 25.3 The three key steps in bone morphogenesis.

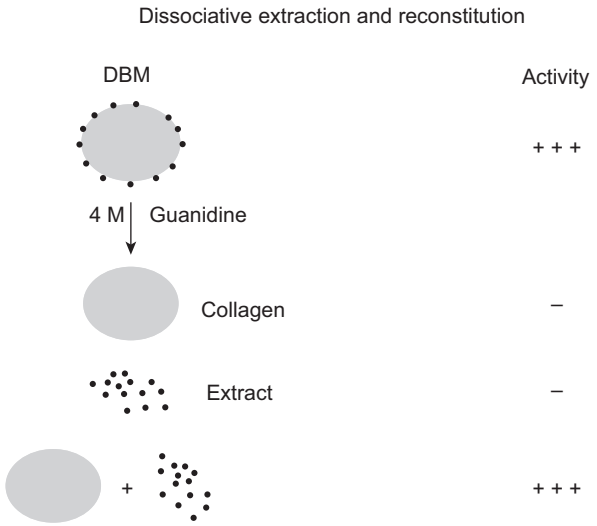

FIGURE 25.4 Dissociative extraction of bone matrix by chaotropic reagents such as 4 M guanidine hydrochloride and reconstitution of the extract with collagenous matrix scaffold. The results indicate that there is a collaboration between the soluble signal in the extract and the insoluble extracellular matrix of bone. *DBM*, demineralized bone matrix.

A major stumbling block in the approach was that the demineralized bone matrix is insoluble and in a solid state. In view of this, dissociative extractants such as 4 M guanidine HCl or 8 M urea as 1% sodium dodecyl sulfate at pH 7.4 were used [11] to solubilize proteins. Approximately 3% of the proteins were solubilized from the demineralized bone matrix; the remaining residue was mainly insoluble type I bone collagen. The extract alone or the residue alone was incapable of new bone induction. However, the addition of the extract to residue (insoluble collagen) and then implantation in a subcutaneous site resulted in bone induction (Fig. 25.4). Therefore, for optimal osteogenic activity it is essential to have a collaboration between the soluble signal in the extract and the insoluble substratum of collagenous ECM [11]. This bioassay was a critical advance in the ultimate purification of BMPs; it led to the determination of limited tryptic peptide sequences and to the eventual cloning of BMPs [12–14]. The dissociative extraction of soluble signals from the demineralized ECM of bone and its subsequent reconstitution with collagen established the cardinal principle of regenerative medicine. The critical principle is that morphogenetic signals stimulate stem cells to differentiate in the optimal scaffold microenvironment (Fig. 25.5). Thus, the triumvirate of signals, stem cells, and scaffolds for regenerative medicine was conceived as a concept.

Although the basic description of bone induction was performed in rats, purification requires a larger and more abundant source of bone. A switch was made to bovine bone. Demineralized bovine bone matrix was not osteoinductive in rats and the results were variable. However, when the guanidine extracts of demineralized bovine bone were fractionated on an S-200 molecular sieve column, fractions less than 50 kD were consistently osteogenic in rats when bioassayed after reconstitution with allogeneic insoluble collagen [15,16]. Thus, protein fractions inducing bone were not species specific and appeared to be homologous in several mammals. It is likely that larger molecular mass fractions and/or the insoluble xenogeneic (bovine and human) collagens were inhibitory or immunogenic. Initial estimates revealed 1 μg of active osteogenic fraction in a kilogram of bone. Hence, over a ton of bovine bone was processed to yield optimal amounts for amino acid sequence determination. The amino acid sequences revealed a homology to transforming growth factor (TGF)-β_1 [16]. The decisive work of Wozney et al.

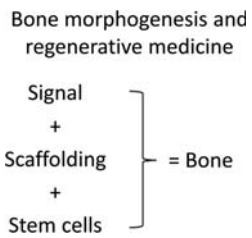

FIGURE 25.5 The key principle of regenerative medicine is that signals stimulate the differentiation of stem cells in the appropriate scaffold.

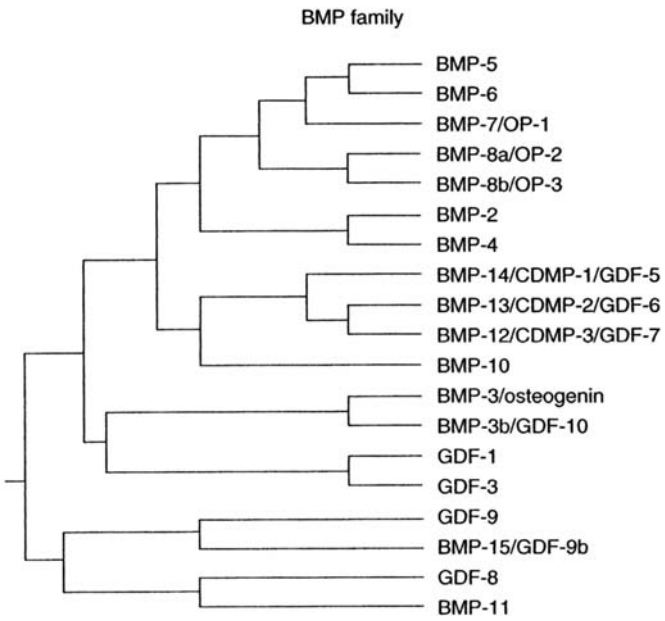

FIGURE 25.6 Members of the bone morphogenetic protein (BMP) family include three main subfamilies: BMP-5, -6, and -7; BMP-2 and -4; BMP-3 and -3b; and growth/differentiation factors (GDF)-5, -6, and -7. *OP*, osteogenic protein.

[12] cloned BMP-2, BMP-2B (now called BMP-4), and BMP-3 (also called osteogenin). Ozkaynak et al. [14] cloned osteogenic proteins 1 and 2 (OP-1 and OP-2). There are several members of this BMP family (Fig. 25.6). Other members of the extended TGF-β/BMP superfamily include inhibins and activins (implicated in follicle-stimulating hormone release from pituitary). Müllerian duct inhibitory substance, growth/differentiation factors (GDFs), nodal, and lefty genes implicated in establishing right and left asymmetry [1,17,18]. BMPs are also involved in embryonic induction [18–21].

BMPs are dimeric molecules, and the conformation is critical for biological actions. Reduction of the single interchain disulfide bond resulted in the loss of biological activity. The mature monomer molecule consists of about 120 amino acids, with seven canonical cysteine residues. There are three intrachain disulfides per monomer and one interchain disulfide bond in the dimer. In the critical core of the BMP monomer is the cysteine knot. The crystal structure of BMP-7 has been determined [22].

Morphogenesis is a sequential multistep cascade. BMPs regulate each of the key steps: chemotaxis, mitosis, and differentiation of cartilage and bone. BMPs initiate chondrogenesis in the limb [23,24]. The apical ectodermal ridge is the source of BMPs in the developing limb bud. The intricate dynamic, reciprocal interactions between the ectodermally derived epithelium and mesoderm-derived mesenchyme sets into motion the train of events culminating in the pattern of the phalanges, radius, ulna, and humerus.

The chemotaxis of human monocytes is optimal at femtomolar concentration [25]. The apparent affinity was 100–200 pM. The mitogenic response was optimal at the 100-pM. range. The initiation of differentiation was in the nanomolar range in solution. However, caution should be exercised because BMPs may be sequestered by ECM components and the local concentration may be higher when BMPs are bounded on the ECM. Thus, BMPs are pleiotropic regulators that act in concentration-dependent thresholds.

It is well-known that ECM components have a critical role in morphogenesis. The structural macromolecules and their supramolecular assembly in the matrix do not explain their role in epithelial–mesenchymal interaction and morphogenesis. This riddle can now be explained by the binding of BMPs to heparan sulfate heparin, and type IV collagen [26–28] of the basement membranes. In fact, this might partly explain the necessity for angiogenesis before osteogenesis during development. In addition, the actions of activin in the development of the frog, in terms of dorsal mesoderm induction, are modified to neutralization by follistatin [29]. Similarly, chordin and noggin from the Spemann organizer induce neutralization by the binding and inactivation of BMP-4. Thus, neural induction is likely to be a default pathway when BMP-4 is nonfunctional [30,31]. This is an emerging principle in development and morphogenesis that binding proteins can terminate a dominant morphogen's action and initiate a default pathway. Finally, the binding of a soluble morphogen to ECM converts it into an insoluble matrix bound morphogen to act locally in the solid state [26].

Although BMPs were isolated and cloned from bone, work with gene knockouts has revealed a plethora of actions beyond bone. Mice with targeted disruption of BMP-2 caused embryonic lethality. The heart development is abnormal, which indicates a need for BMP-2 in heart development [32]. BMP-4 "knockouts" exhibit no mesoderm induction and gastrulation is impaired [33]. Transgenic overexpression of BMPs under the control of keratin 10 promoter leads to psoriasis. The targeted deletion of BMP-7 revealed the critical role of this molecule in kidney and eye development [34–36]. Thus, the BMPs are really true morphogens for such disparate tissues as skin, heart, kidney, and eye. In view of the emerging wider role, BMPs may be called bone morphogenetic proteins (BMPs).

Recombinant human BMP-4 and BMP-7 bind to BMP receptor IA (BMPR-IA) and BMP receptor IB (BMPR-IB) [37]. CDMP-1 also binds to both of the type I BMP receptors. There is a collaboration between type I and II BMP receptors [38]. The type I receptor serine/threonine kinase phosphorylates a signal-transducing protein substrate called Smad 1 or 5 [39]. Smad is a term derived from fusion of Drosophila Mad gene and *Caenorhabditis elegans* (nematode) Sma gene. Smads 1, 5, and 8 signal in partnership with a common co-Smad, Smad 4 (Fig. 25.7). The transcription of BMP-response genes is initiated by Smad 1/Smad 4 heterodimers. Smads are trimeric molecules as gleaned by X-ray crystallography. The phosphorylation of Smads 1 and 5 by type I BMP receptor kinase is inhibited by inhibitory Smad 6 and 7 [40]. Smad interacting protein may interact with Smad 1 and modulate BMP-response gene expression [18,41]. The downstream targets of BMP signaling are likely to be homeobox genes, the cardinal genes for morphogenesis and transcription. BMPs, in turn, may be regulated by members of the hedgehog family of genes such as Sonic and Indian hedgehog [42].

STEM CELLS

It is well-known that embryonic mesoderm-derived mesenchymal cells are progenitors for bone, cartilage, tendons, ligaments, and muscle. However, certain stem cells in adult bone marrow, muscle, and fascia can form bone and cartilage (Fig. 25.8). The identification of stem cells readily sourced from bone marrow may lead to banks of stem cells for cell therapy and perhaps gene therapy with appropriate "homing" characteristics to bone marrow and hence to skeleton. The pioneering work of Friedenstein et al. [43,44], and Owen and Friedenstein [45] identified bone marrow stromal stem cells. These stromal cells are distinct from the hematopoietic stem cell lineage. Bone marrow stromal stem cells consist of inducible and determined osteoprogenitors committed to osteogenesis. Determined osteogenic precursor cells have the propensity to form bone cells with no external cues or signals. On the other hand, inducible osteogenic precursors require an inductive signal such as BMP or demineralized bone matrix. The operational distinction between stromal stem cells and hematopoietic stem cells is becoming increasingly less distinct. The stromal stem cells of Friedenstein and Owen are also called mesenchymal stem cells [46,47], with the potential to form bone, cartilage, adipocytes, and myoblasts in response to cues from the environment and/or intrinsic factors. Mesenchymal stem cells are present in synovium [48], periosteum [49], adipose tissue [50], and blood [51]. There is considerable hope and anticipation that these bone marrow stromal cells may be excellent vehicles for cell and gene therapy [52,53].

From a practical standpoint, these stromal stem cells can be obtained by bone marrow biopsies and expanded rapidly for use in cell therapy after pretreatment with BMPs. Their potential use in both cell and gene therapy is promising. There are continuous improvements in the viral vectors and efficiency of gene therapy [54–57]. For example, it is possible to use BMP genes transfected in stromal stem cells for targeting to bone marrow.

SCAFFOLDS OF EXTRACELLULAR MATRIX AND BIOMIMETIC BIOMATERIALS

The earlier discussion regarding inductive signals (BMPs) and responding stem cells (stromal cells) leads us to the scaffolding (the microenvironment and ECM) for optimal tissue engineering. Natural biomaterials in the composite tissue of bones and joints are collagens, proteoglycans, and glycoproteins of cell adhesion such as fibronectin and the mineral phase. The mineral phase in bone is predominantly hydroxyapatite. In their native state, the associated citrate, fluoride, carbonate, and trace elements constitute physiological hydroxyapatite. The high protein binding capacity makes hydroxyapatite a natural delivery system. Comparison of insoluble bone collagenis an optimal delivery system for BMPs [58]. It is well-known that collagen is an ideal delivery system for growth factors in soft and hard tissue wound repair [59].

During the course of systematic work on hydroxyapatite of two pore sizes (200 or 500 μm) in two geometrical forms (beads and disks), an unexpected observation was made. The geometry of the delivery system is critical

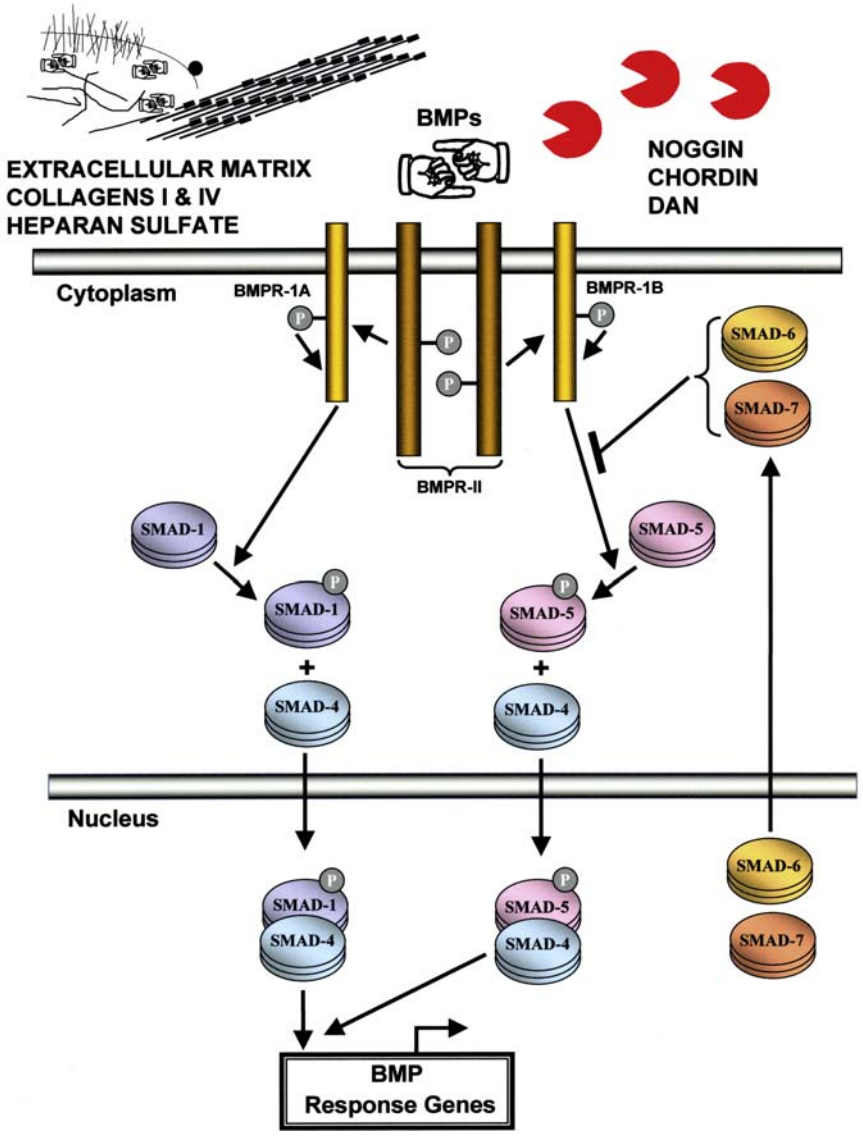

FIGURE 25.7 Bone morphogenetic protein (BMP) receptors (BMPR) and signaling cascades. BMPs are dimeric ligands with a cysteine knot in each monomer fold. Each monomer has two β sheets represented as two pointed fingers. In the functional dimer, the fingers are oriented in opposite directions. BMPs interact with type I and II BMPR. The exact stoichiometry of the receptor complex is being elucidated. BMPR-II phosphorylates the GS domain of BMPR-I. The collaboration between type I and II receptors forms the signal-transducing complex. BMP type I receptor kinase complex phosphorylates the trimeric signaling substrates Smad 1 or Smad 5. This phosphorylation is inhibited and modulated by inhibitory Smads 6 and 7. Phosphorylated Smad 1 or 5 interacts with Smad 4 (functional partner) and enters the nucleus to activate the transcriptional machinery for early BMP response genes. A novel SIP may interact and modulate the binding of heteromeric Smad 1/Smad 4 complexes to the DNA.

for optimal bone induction. The disks were consistently osteoinductive with BMPs in rats, but the beads were inactive [60]. The chemical composition of the two hydroxyapatite configurations was identical. In certain species, the hydroxyapatite alone appears to be "osteoinductive" [61]. In subhuman primates, the hydroxyapatite induces bone, albeit at a much slower rate. One interpretation is that osteoinductive endogenous BMPs in circulation progressively bind to the implanted disk of hydroxyapatite. When an optimal threshold concentration of native BMPs is achieved, the hydroxyapatite becomes osteoinductive. Strictly speaking, most hydroxyapatite substrata are ideal osteoconductive materials. This example in certain species also serves to illustrate how an osteoconductive biomimetic biomaterial may progressively function as an osteoinductive substance by binding to endogenous BMPs. Thus, there is a physiological–physicochemical continuum between the hydroxyapatite alone and progressive

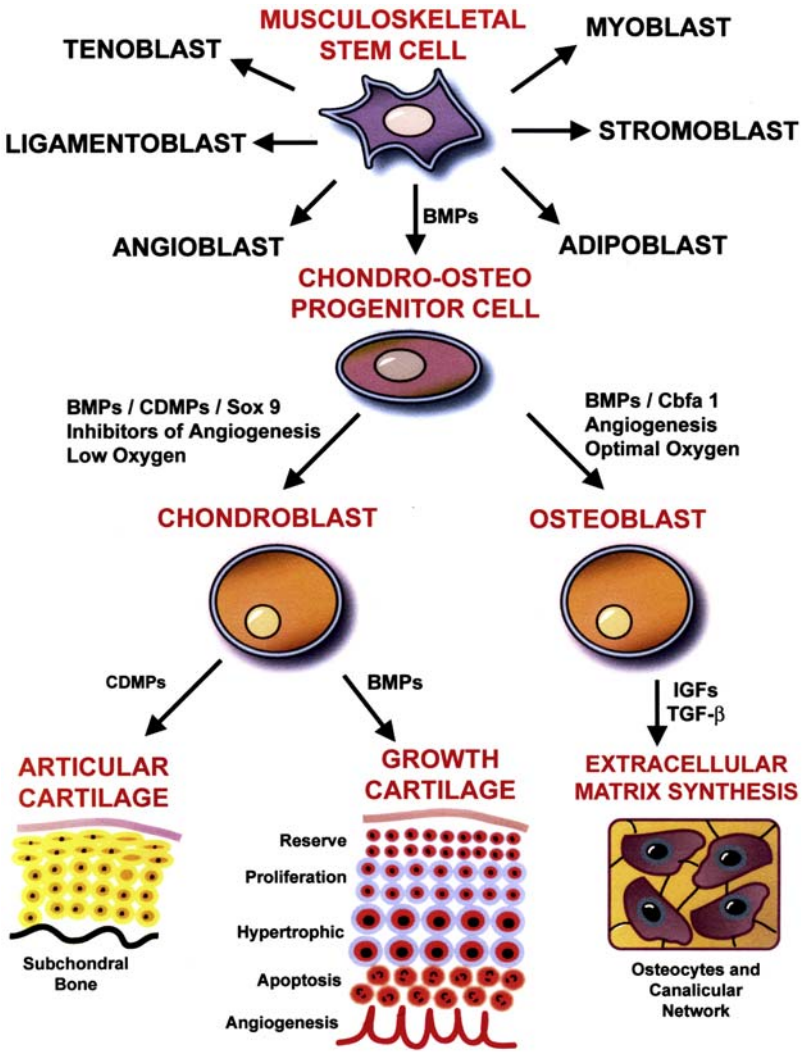

FIGURE 25.8 The lineage of the putative musculoskeletal stem cell. The bone morphogenetic proteins (BMPs) determine the lineage into chondro/osteoprogenitor cells and further specialization into articular chondrocytes growth plate chondrocytes and osteoblast lineage. BMPs are morphogens critical for directing the differentiation of cartilage and bone cells. *Cbfa 1*, core-binding factor α(1); *CDMP*, cartilage-derived morphogenetic proteins; *IGF*, insulin-like growth factor; *TGF-β*, transforming growth factor- *β*.

composites with endogenous BMPs. Recognition of this experimental nuance will save unnecessary arguments among biomaterials scientists about the osteoinductive action of a conductive substratum such as hydroxyapatite.

Complete regeneration of a baboon craniotomy defect was accomplished by recombinant human osteogenic protein (human BMP-7) [62]. Recombinant BMP-2 was delivered by a poly(hydroxy acid) carrier for calvarial regeneration [63]. Copolymer of polylactic and polyglycolic acid was evaluated as a delivery system for BMP 2 in a rabbit ulna nonunion model and the results were satisfactory (Fig. 25.9) [64].

An important problem in the clinical application of biomimetic biomaterials with BMPs and/or other morphogens in regenerative medicine is sterilization. Although gas (ethylene oxide) is used, one should always be concerned about reactive free radicals. Using allogenic demineralized bone matrix with endogenous native BMPs, as long as a low temperature is maintained (4°C or less), the samples tolerate up to 5–7 milliradians of irradiation [65,66]. The standard dose acceptable to the US Food and Drug Administration (FDA) is 2.5 milliradians. This information is useful to biotechnology companies preparing to market recombinant BMP-based osteogenic devices. Perhaps a tissue banking industry with an interest in bone grafts [67] may also use this critical information. Various freeze-dried and demineralized allogeneic bones may be used in the interim as satisfactory carriers for BMPs. The moral of this experiment is that it is not the irradiation dose but the ambient sample temperature during irradiation that is absolutely critical.

FIGURE 25.9 Bone morphogenetic proteins (BMPs) have wide-ranging roles in regenerative medicine and surgery. Their applications include, but are not limited to, orthopedics and plastic and reconstructive surgery, in dentistry and oral surgery. Recombinant BMP-2 has been approved by the US Food and Drug Administration for spine fusion and the nonunion of fractures.

ARTICULAR CARTILAGE REGENERATION AND CARTILAGE MORPHOGENETIC PROTEINS

Morphogenesis of the cartilage is the most important rate-limiting step in the dynamics of bone development. Cartilage is the initial model for the architecture of bones. Bone can form either directly from mesenchyme, as in intramembranous bone formation, or with an intervening cartilage stage, as in endochondral bone development [7]. All BMPs first induce the cascade of chondrogenesis; therefore, they all sense cartilage morphogenetic proteins. The hypertrophic chondrocytes in the epiphyseal growth plate mineralize and serves as a template for appositional bone morphogenesis. Cartilage morphogenesis is critical for both bone and joint morphogenesis. The two lineages of cartilage are clear-cut. The first, at the ends of bone, forms articulating articular cartilage. The second is the growth plate chondrocytes, which under hypertrophy and synthesize cartilage matrix destined to calcify before replacement by bone and are the "organizer" centers of longitudinal and circumferential growth of cartilage, setting into motion the orderly program of endochondral bone formation. The phenotypic stability of articular (permanent) cartilage is at the crux of the problem of osteoarthritis. The "maintenance" factors for articular chondrocytes include TGF-β isoforms and the BMP isoforms [68].

An in vivo chondrogenic bioassay with soluble purified proteins and insoluble collagen scored for chondrogenesis. A concurrent reverse transcription–polymerase chain reaction approach was taken with degenerate oligonucleotide primers. Two novel genes for CDMPs 1 and 2 were identified and cloned [69]. CDMPs 1 and 2 are also called GDF-5 and GDF-6, respectively [70]. CDMPs are related to BMPs (Fig. 25.6). CDMPs are critical for cartilage and joint morphogenesis [71]. CDMPs stimulate proteoglycan synthesis in cartilage. GDF-7 initiates tendon and ligament morphogenesis.

REGENERATIVE MEDICINE AND SURGERY OF ARTICULAR CARTILAGE

Unlike bone, with its considerable prowess for repair and constant regeneration, articular cartilage is recalcitrant. The recalcitrant regeneration in articular cartilage may be due to the relative avascularity of the tissue, the high concentration of protease inhibitors, and perhaps even cytokine inhibitors. The wound debridement phase is not optimal for preparing the cartilage wound bed for best possible regeneration. Although cartilage has been successfully engineered to predetermined shapes [72], true repair of the tissue continues to be a real challenge in part because of hierarchical organization and geometry [73]. The use of autologous culture-expanded human chondrocytes is expanding [74]. Also gaining increasing attention is mosaicplasty for defects in articular cartilage [75]. A continuous challenge in chondrocyte cell therapy is progressive dedifferentiation and loss of a characteristic cartilage phenotype. The redifferentiation and maintenance of chondrocytes for cell therapy can be aided by BMPs, CDMPs, TGF-β isoforms, and insulin growth factors. It is also possible to repair cartilage using muscle-derived mesenchymal stem cells [76]. The possibility of problems posed by cartilage proteoglycans in preventing cell immigration for repair was investigated by chondroitinase ABC and trypsin pretreatment in partial-thickness defects [77] with and without TGF-β. Pretreatment with chondroitinase ABS followed by TGF-β revealed a contiguous layer of cells from the synovial membrane that hinted at the potential source of "repair" cells from synovium. Multiple avenues of cartilage morphogens, cell therapy with chondrocytes, and stem cells from marrow and muscle and a biomaterial scaffolding may lead to optimal tissue-engineered articular cartilage.

REGENERATION OF ARTICULAR CARTILAGE SURFACE AND LUBRICATION

The articular cartilage is a tissue that functions in weight bearing and in friction-free locomotion. Unlike bone, the articular cartilage is recalcitrant to repair and regeneration. The articular cartilage is avascular. Because of this, the innate mechanism of tissue regeneration is feeble and the recruitment of stem and progenitor cells is impaired. In addition, untreated injury to the cartilage progresses to osteoarthritis, with pain and degenerative joint tissue damage [78]. Severe pain is associated with the clinical symptoms of osteoarthritis, and there is a financial burden. In the articular cartilage, regeneration of the superficial zone is critical to maintain joint mobility and reduce wear by optimizing lubrication. The chondrocytes in the superficial zone secrete superficial zone protein (SZP), also known as lubricin and proteoglycan 4, which is encoded by the *prg4* gene. SZP has an important role in lubrication as a boundary lubricant and joint homeostasis. Three key ingredients for the regeneration of the surface of the articular cartilage are signals, stem cells, and scaffolds. SZP secreted by the surface is critical for lubrication. Partial-thickness defects in the articular cartilage do not heal. On the other hand, full-thickness defects penetrating the subchondral bone permit the healing of cartilage. Three important elements for both morphogenesis and regeneration are signals for morphogenesis, stem cells, and scaffolds of ECM. Regeneration is essentially a recapitulation of the sequential cascade of embryonic development and morphogenesis [1].

Normal articular cartilage maintains a functionally optimized lubricated surface with a low coefficient of friction to reduce wear and preserve joint mobility [79]. Cartilage lubrication is critical for joint mobility. Experiments in animals have demonstrated that SZP reduces cartilage degeneration after knee injury [80]. Autologous platelet-rich plasma (PRP) has been used to ameliorate pain, based on the presence of platelet-derived growth factor in the PRP. In an investigation, human PRP significantly stimulated cell proliferation and SZP secretion by surface chondrocytes of the articular cartilage and synoviocytes from the knee joint. In addition, PRP stimulated lubrication in bovine articular cartilage superficial zone in a functional bioassay [79].

It is inevitable during aging most humans will confront the challenges of impaired locomotion owing to wear and tear in bones and joints. Therefore, repair and possibly complete regeneration of the musculoskeletal system and other vital organs such as skin, liver, and kidney may need optimal repair or a spare part for replacement. Can we create spare parts for the human body? There is much reason for optimism that tissue engineering can help patients. We are living in an extraordinary time for biology, medicine, surgery, and computational and related technology. The confluence of advances in developmental biology and inductive signals for morphogenesis of articular cartilage may lead to quantum advances in tissue engineered joints.

The symbiosis of biotechnology and biomaterials has set the stage for systematic advances in tissue engineering [16,81,82]. Advances in enabling platform technology include molecular imprinting [83]. In principle, specific recognition and catalytic sites are imprinted using templates. Applications range from biosensors to catalytic applications, antibody, and receptor recognition sites. For example, the cell-binding RGD site in fibronectin [84] or YIGSR domain in laminin can be imprinted in complementary sites [85].

The rapidly advancing frontiers in morphogenesis with BMPs, hedgehogs, homeobox genes, and a veritable cornucopia of general and specific transcription factor coactivators and repressors will lead to cocrystallization of ligand−receptor complexes, protein−DNA complexes, and other macromolecular interactions. This will lead to peptidomimetic agonists for large proteins, as exemplified by erythropoietin [86]. To such advances one can add new developments in the self-assembly of millimeter-scale structures floating at the interface of perfluorodecalin and water and interacting by capillary forces controlled by the pattern wettability [87]. The final self-assembly is caused by the minimization of free energy in the interface. These are truly incredible advances that will lead to man-made materials that mimic ECM in tissues. Now, superimpose on such chemical progress a biological platform in a bone and joint mold. Let us imagine a head of the femur: a mold is fabricated with computer-assisted design and manufacture. It faithfully reproduces the structural features and may be imprinted with morphogens, inductive signals, and cell adhesion sites. This assembly can be loaded with stem cells and BMPs and other inductive signals with a nutrient medium optimized for optimal number of cell cycles, and then predictably exit into differentiation phase to reproduce a totally new bone femoral head. In fact, such a biological approach with a vascularized muscle flap and BMPs has yielded new bone with a defined shape and has demonstrated the proof of principle for further development and validation [88]. We are indeed entering a brave new world of prefabricated biological spare parts for the human body based on sound architectural rules of inductive signals for bone morphogenesis and responsive stem cell lineage control by BMPs, and with growth and differentiation factors immobilized on a template of biomimetic biomaterials based on ECM.

List of Acronyms and Abbreviations

ECM Extracellular matrix
BMPs Bone morphogenetic proteins
CDMPs Cartilage-derived morphogenetic proteins
BGP Bone gla protein
SDS Sodium dodecyl sulfate
TGF Transforming growth factor
OP-1 and OP-2 Osteogenic proteins 1 and 2
MIS Müllerian duct inhibitory substance
GDFs Growth/differentiation factors
BMPR-IA BMP receptor IA
BMPR-IB BMP receptor IB
SIP Smad interacting protein
rhOP-1 Recombinant human osteogenic protein
FDA Food and Drug Administration
RT-PCR Reverse transcription-polymerase chain reaction
IGFs Insulin growth factors
SZP Superficial zone protein
PRP Platelet-rich plasma
PDGF Platelet-derived growth factor

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CHAPTER

26

Physical Stress as a Factor in Tissue Growth and Remodeling

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INTRODUCTION

The goal of tissue engineering and regenerative medicine is to restore or regenerate damaged and degenerate tissues, many of which have explicitly mechanical functions or are regulated by mechanical factors. Indeed, physical stimuli are essential for the proper morphogenesis, maintenance, and repair of numerous tissue types, including bone, cartilage, and blood vessels, all of which are primary targets for tissue engineers. Therefore, being able to describe and understand the role of physical stress and strain mathematically in tissue repair is essential to regenerating functional, mechanically competent tissues.

The role of physical stresses and strains in regulating tissue growth and remodeling has been tremendously interesting to investigators for well over 100 years. Although unfairly to his contemporary colleagues, Julius Wolff is often credited with the concept that tissue structure or form follows from its function (i.e., Wolff's law). At the time, Wolff's law was simply based on the apparent correspondence between anatomical observations of trabecular bone organization and estimations of principal stress directions caused by functional loading conditions. The recognition that adaptation of tissue structure and composition is cell-mediated was not made until later by other investigators. These early observations spawned the interdisciplinary field of mechanobiology, which is focused on identifying mechanisms by which mechanical signals are transduced into cellular activity; it emphasizes the need to consider the effects of physical factors on tissue growth and remodeling as an important part of strategies for tissue regeneration.

Many different cell types from various tissues have been shown to be sensitive to mechanical stimuli in one form or another. The effects of physiological mechanical signals on cells and tissues can be beneficial; they have a central role in maintaining tissue structural integrity via remodeling processes. Alterations in mechanical signals can also contribute to the development of pathological conditions. For example, local shear stresses have a key role in developing and localizing atherosclerotic lesions. Likewise, the progression of osteoarthritis is caused by a vicious cycle of cartilage matrix degradation and increased local stresses. In bone, the mechanical environment also has important clinical implications in the development of osteoporosis, stress fractures, total joint implant loosening, and bone loss during space flight.

Therefore, approaching a tissue-engineering problem from a mechanical perspective involves four steps: (1) describe the native mechanical environment quantitatively, (2) understand the role of mechanical factors in the tissue of interest, (3) manipulate mechanical conditions to enhance function or regeneration, and (4) evaluate the degree of functional restoration of the engineered tissue quantitatively. This chapter will present an overview of the tools and concepts required in each.

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DESCRIBE THE MECHANICAL ENVIRONMENT

As mentioned, cells respond and adapt to their local mechanical environment, but to understand this phenomenon, the mechanical environment must be usefully described. This requires an appropriate theoretical framework that can accurately model the salient features of the tissue of interest and describe the local mechanical stimuli experienced by a cell either in situ or in a tissue-engineered construct. The framework must therefore describe the properties and behavior of the tissue or construct, incorporate the loads (boundary conditions) that will be applied, and justify the assumptions made.

It is useful to view tissues as a structural hierarchy through which functional loads are transmitted down to the cellular level (Fig. 26.1). In bone, for example, applied joint and muscle forces result in stresses and strains within the mineralized tissue that can be defined at different scale levels, from the whole-bone level down to submicron mineral crystals embedded within collagen molecules. At each hierarchical level, it is convenient to assume that everything below that level is a continuum (i.e., there is a finite mass density at every point within the material). This simplification allows material properties to be expressed at a given hierarchical level in terms of constitutive equations. As described in the next section, constitutive equations define the relationship between stresses and strains at each level. Cells sense and respond to local stresses or strains produced by forces transmitted from the macrolevel down through the complex structural hierarchy to the cellular level. Cell-mediated adaptational changes in tissue structure and composition subsequently alter the local stresses and strains resulting from functionally applied loads, thus providing a regulatory feedback mechanism. The sensitivity of the cellular response to mechanical stimuli can be altered by a variety of nonmechanical factors such as age and disease, as well as numerous biochemical factors.

Strain and Stress Definitions

Strain

Strain is a normalized measure of deformation. Consider the simple case of a thin rectangular piece of tissue being axially loaded by a force, as shown in Fig. 26.2A. The axial force increases the length of the tissue, but at the same time it decreases its width and thickness. Engineering strain is defined as the change in a dimension of the tissue normalized by its original dimension and is given in the axial direction by:

$$\varepsilon_{11} = \frac{L - L_0}{L_0} \tag{26.1}$$

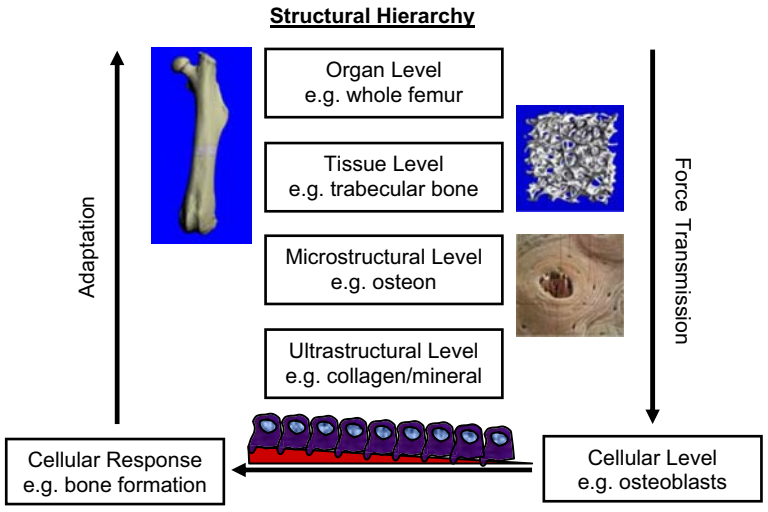

FIGURE 26.1 Force transmission through the structural hierarchy of bone to the cellular level resulting in cell-mediated adaptation of tissue structure and composition.

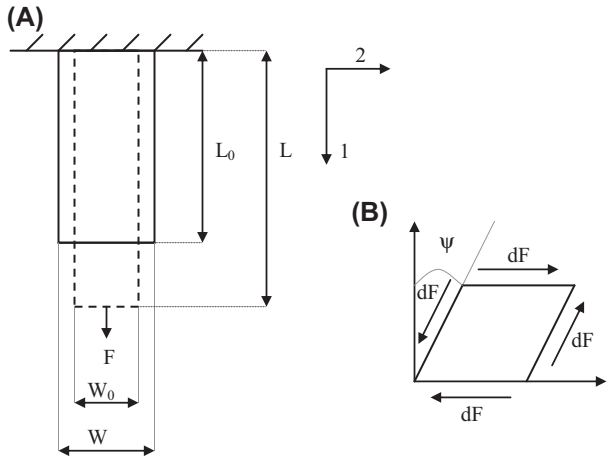

FIGURE 26.2 (A) Axial and transverse strains associated with uniaxial tensile loading. (B) Shear strain associated with torsional or shear loading.

Another important deformation parameter is the Poisson ratio ν, which is defined as the ratio of lateral strain to axial strain and in this case is given by:

$$\nu = -\frac{\varepsilon_{22}}{\varepsilon_{11}} = -\frac{\dfrac{W - W_0}{W_0}}{\dfrac{L - L_0}{L_0}} \tag{26.2}$$

The Poisson ratio is a measure of the tendency for a material body to try to retain its total volume as it is deformed. When $\nu = 0.5$, the material is said to be incompressible (e.g., water) and does not undergo a volume change after deformation. The typical value of ν for tissues is between 0.2 and 0.45. Thus, a tissue subjected to tensile deformation and strain would slightly increase in volume. In contrast to normal strains, shear strains caused by simple shear forces, dF, or from torsional loading, for example, produce a change in shape but not volume, as shown in Fig. 26.2B. Measurement of the angle of shear deformation, γ_{12}, allows calculation of shear strain, as given by:

$$\varepsilon_{12} = \frac{\gamma_{12}}{2} \tag{26.3}$$

The complex deformations created by forces acting in multiple directions necessitate the generalization of deformation to three-dimensional (3-D) space. Deformation in 3-D can be expressed by the deformation gradient tensor, **F**. Consider the body shown in Fig. 26.3A undergoing a deformation from the reference state to a deformed configuration. If one follows the particles P_1 and P_2, they move from position $\mathbf{X}_{P1}$ and $\mathbf{X}_{P2}$ to $\mathbf{x}_{P1}$ and $\mathbf{x}_{P2}$, respectively. There will also be a similar one-to-one mapping of other particles in the reference and deformed configurations. Thus, the deformation of the body can be written as a function relating the current state (lowercase) to the reference state (uppercase):

$$\mathbf{x} = \mathrm{f}(\mathbf{X}) \tag{26.4}$$

In scalar form, this would involve three equations:

$$\begin{aligned} x_1 &= f_1(X_1, X_2, X_3), \\ x_2 &= f_2(X_1, X_2, X_3), \\ x_3 &= f_3(X_1, X_2, X_3), \end{aligned} \tag{26.5}$$

where 1, 2, and 3 correspond to the three directions of the coordinate system.

The displacement vector is given by:

$$\mathbf{u} = \mathbf{x} - \mathbf{X} \tag{26.6}$$

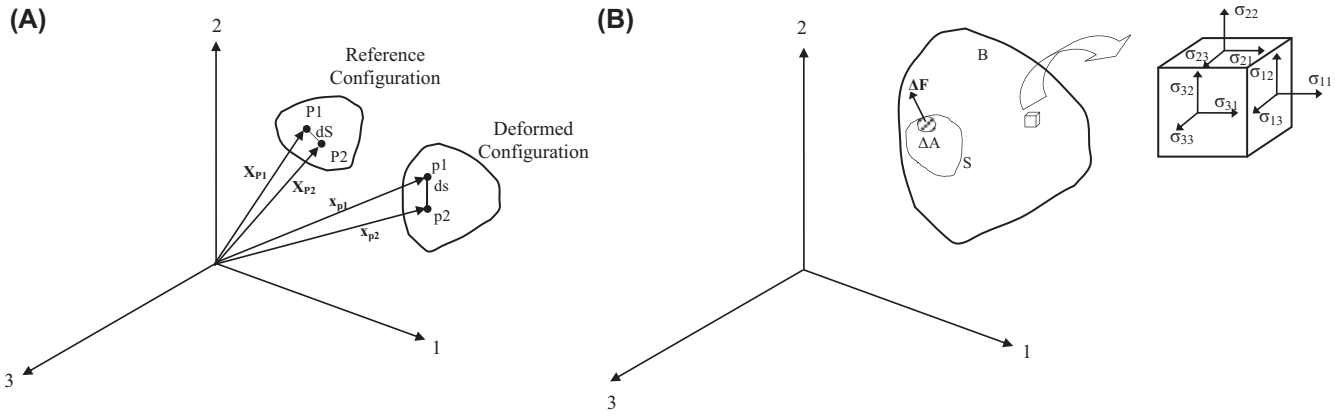

FIGURE 26.3 (A) Deformation of a 3D body from a reference configuration to a deformed configuration. (B) Stress on a surface element, and the nine stress components defining the stress state at a point.

The deformation gradient **F** is then defined as:

$$\mathbf{F} = \frac{\partial \mathbf{x}}{\partial \mathbf{X}} \tag{26.7}$$

In matrix form, the deformation gradient can be written as:

$$[\mathrm{F}] = \begin{bmatrix} \frac{\partial x_1}{\partial X_1} & \frac{\partial x_1}{\partial X_2} & \frac{\partial x_1}{\partial X_3} \\ \frac{\partial x_2}{\partial X_1} & \frac{\partial x_2}{\partial X_2} & \frac{\partial x_2}{\partial X_3} \\ \frac{\partial x_3}{\partial X_1} & \frac{\partial x_3}{\partial X_2} & \frac{\partial x_3}{\partial X_3} \end{bmatrix}, \tag{26.8}$$

and is related to the gradient of displacement by the following expression, in which **I** is the identity tensor:

$$\mathbf{F} = \frac{\partial \mathbf{u}}{\partial \mathbf{X}} + \mathbf{I} \tag{26.9}$$

It is appropriate to use the engineering strains as just defined when the strains in the material are small (typically less than 5%). However, the analysis of large deformations, as frequently observed for soft tissues under functional loading conditions, requires the use of other strain measures. When the deformation is large, a useful measure of deformation is the Green (or Lagrangian) strain, **E**, which is defined as:

$$\mathbf{E} = \frac{1}{2}\left[\mathbf{F}^{\mathrm{T}}\mathbf{F} - \mathbf{I}\right] = \frac{1}{2}\left[\mathbf{D} + \mathbf{D}^{\mathrm{T}} + \mathbf{D}^{\mathrm{T}}\mathbf{D}\right], \tag{26.10}$$

where $\mathbf{D} = \frac{\partial \mathbf{u}}{\partial \mathbf{X}}$ and superscript "T" stands for the transpose of the matrix form of the second-order tensor.

Consider the segment P_1P_2 of length dS that has deformed to p_1p_2 with length ds. In this case, the 1-D Green strain becomes:

$$\mathrm{E} = \frac{1}{2}\left(\frac{ds^2 - dS^2}{dS^2}\right) \tag{26.11}$$

If the deformation under consideration is small, as is typically the case for bone and most structural engineering materials, the quadratic term, $\mathbf{D}^{\mathrm{T}}\mathbf{D}$ in the Green strain can be neglected to give the infinitesimal (engineering) strain tensor, $\boldsymbol{\varepsilon}$:

$$\boldsymbol{\varepsilon} = \frac{1}{2}\left[\mathbf{D} + \mathbf{D}^{\mathrm{T}}\right], \tag{26.12}$$

which in 1-D gives us the familiar expression of engineering strain in a uniaxial test:

$$\varepsilon = \frac{\mathrm{L} - \mathrm{L}_0}{\mathrm{L}_0}. \tag{26.13}$$

To begin to understand the relative values of these strain measures, consider the following example of uniaxial elongation of our rectangular tissue with an original length of 5 cm. In one case, the tissue is stretched to a final length of 5.05 cm (small strain), whereas in the second case, it is elongated to 10 cm (large strain).

	Case I (L = 5.05 cm)	Case II (L = 10 cm)
Green strain $\left(E = \frac{1}{2}\frac{L^2 - L_0^2}{L_0^2}\right)$	0.01005	1.5
Engineering strain $\left(\varepsilon = \frac{L - L_0}{L_0}\right)$	0.01	1.0

Thus, we see that for the small deformations, the different strain definitions give approximately the same value and engineering strain is reasonably accurate, whereas for large deformations, the strain definitions yield different values owing to neglect of the higher-order terms in the engineering strain definition.

Stress

Stress is a measure of the intensity of internal force developed in a material upon application of an external force. Consider force $\Delta\mathbf{F}$ acting on a small surface element of area ΔA in Fig. 26.3B. This element lies on the surface S, which is part of the larger body B. As ΔA tends to zero, the ratio $\frac{\Delta F}{\Delta A}$ tends to a finite limit $\frac{dF}{dA}$, which is defined as the stress on the surface element.

Consider an infinitesimal cube in the body, as shown in Fig. 26.3B. Because of the external force applied on the body, internal forces are applied on the surface of the cube. Each internal force can be resolved into its three components and normalized by the area to give three stress components on each face. The volume of the cube can be continuously decreased such that the cube collapses to a point. The nine stress components define the second-order stress tensor and completely describe the stress state at this point. Using equilibrium conditions, we can show that the stress tensor is symmetric: that is, $\sigma_{ij} = \sigma_{ji}$; thus, the stress tensor has only six independent components. If a stress component acts in a direction perpendicular to the surface on which it acts, it is referred to as a normal stress. On the other hand, if it is parallel to the surface, it is called a shear stress. Thus, σ_{11}, σ_{22}, and σ_{33} are normal stresses, whereas $\sigma_{12,}$ σ_{23}, and σ_{31} are shear stresses. Normal stresses can change both the volume and shape of the body, whereas shear stresses induce only shape change.

The first Piola–Kirchoff stress tensor, $\mathbf{P}$, is defined as the force acting in the current configuration, $\Delta\mathbf{F}$, on an oriented area in the reference configuration, ΔA, in the limit as ΔA tends to zero. In a typical experiment, the force is constantly measured but the cross-sectional area is not. Thus, the first Piola–Kirchoff stress is an easy quantity to compute because the undeformed cross-sectional area can be measured before loading. However, when considering the balance of forces in the deformed body at equilibrium, the deformed area Δa of the surface element is required for the stress definition. The Cauchy stress, or "true" stress, is thus defined as the limit of $\sigma = \frac{\Delta\mathbf{F}}{\Delta a}$ as Δa tends to zero. Although the difference between Δa and ΔA is negligible for small deformations, the difference becomes significant for large deformations and choice of stress definition is important. A third definition, the second Piola–Kirchoff stress tensor, $\mathbf{S}$, is often used in constitutive modeling and is defined as the limit of $\mathbf{S} = \frac{\Delta\mathbf{F}'}{\Delta A}$ as ΔA tends to infinity, where $\Delta\mathbf{F}'$ is the current force, $\Delta\mathbf{F}$, mapped onto the reference configuration and therefore is defined completely in terms of the reference state. All three stress definitions can be related to one another by the deformation gradient [1].

Constitutive Relations

These definitions enable us to begin establishing a theoretical framework in which to describe the material behavior of the tissue of interest in response to mechanical forces. The material behavior will be expressed as a constitutive equation, a mathematical model that specifies the relationship between stress and strain. At this stage, assumptions must be made regarding the behavior of the material, which will dictate the framework selected. These assumptions will be tested by experimental validation of the model. The goal of constitutive modeling is to describe the important features of a tissue's material behavior in the simplest and most mathematically useful way possible. For example, the simplest constitutive equation is that for linearly elastic, homogeneous, isotropic materials, in which there is a linear, reversible relationship between stress and strain and the material properties do not vary by position or direction. For such materials, we are limited to discussion of small deformations. This gives the familiar equation, $\sigma = \mathbf{E}\,\varepsilon$, in which E is the elastic modulus and σ and ε are the engineering stress and strain, respectively. Many engineering materials (i.e., steel) can be modeled in this way; however, most biological materials are more complex.

Mechanically speaking, bone is one of the simpler tissue types and can be modeled as linear elastic, although it is highly inhomogeneous and anisotropic (the properties vary by both position and direction). This behavior can be described well using the generalized Hooke law, which can be written in indicial notation as:

$$\sigma_{ij} = C_{ijkl}\varepsilon_{kl}, \tag{26.14}$$

where C_{ijkl} is a fourth-order tensor describing the material properties and contains 81 constants. However, owing to symmetry arguments and thermodynamic constraints, the number of independent constants is reduced to 21. If the six stress and strain components are written in the form of a column matrix, the material tensor can be represented by a matrix called the stiffness matrix:

$$C = \begin{bmatrix} C_{11} & C_{12} & C_{13} & C_{14} & C_{15} & C_{16} \\ & C_{22} & C_{23} & C_{24} & C_{25} & C_{26} \\ & & C_{33} & C_{34} & C_{35} & C_{36} \\ & & & C_{44} & C_{45} & C_{46} \\ & & & & C_{55} & C_{56} \\ & & & & & C_{66} \end{bmatrix}, \tag{26.15}$$

where the other side of the diagonal is symmetric (i.e., $C_{ij} = C_{ji}$). This stiffness matrix represents a fully anisotropic linear elastic material for which 21 constants must be determined experimentally to characterize the material behavior fully. Fortunately, many tissues have some degree of material symmetry. For example, trabecular bone is frequently described using an orthotropic material model, which consists of three mutually orthogonal planes of symmetry that coincide with the chosen reference coordinate system. This reduces the numbers of independent constants to nine, which are related to the Young's moduli (E_1, E_2, and E_3) and shear moduli (G_1, G_2, and G_3) and the Poisson ratios (ν_{12}, ν_{23}, and ν_{31}) in the three planes. In the case of a fully isotropic material, which has infinite planes of symmetry, these equations reduce to the basic mechanics of materials expression of the Hooke law, with only two independent constants, E and ν.

For most biological materials (e.g., cartilage, tendon, blood vessels) the constitutive models must describe an expanding array of features. Unlike most engineering materials, most tissues are nonlinear, viscoelastic (i.e., time-dependent), inhomogeneous, and anisotropic, and experience large deformations under physiologic loads. It can be difficult to include every one of these features in a 3-D model. Fortunately, reasonable assumptions can be made to simplify the approach and yield useful information about the most important features of the tissue at hand. For example, Y.C. Fung made the observation that in many soft tissues, after several "preconditioning" cycles, the loading and unloading curves reach a steady state and the final curves lose much of their rate dependence [2]. He termed this characteristic behavior "pseudoelasticity." Thus, many tissues such as skin, tendon, and blood vessels can often be modeled as nonlinear, pseudoelastic, homogeneous, and anisotropic, with large deformations. Using these assumptions and applying the second law of thermodynamics (via the Clausius–Duhem entropy inequality), it can be shown that the second Piola–Kirchoff stress, **S**, is directly related to the derivative of the strain potential, or strain energy function, W, with respect to the Green strain, **E**:

$$S = \rho_0 \frac{\partial W}{\partial E}, \tag{26.16}$$

where ρ_0 is the mass density. Now, provided we know a proper strain energy function for a given material, we have a direct relationship between the stress and the strain. Selection of a proper functional form for W is an active area of research and can account for anisotropy, incompressibility, and nonlinearity [2,3]. One commonly used model, proposed by Fung [4,5], assumes that the exponential relationship observed in 1-D can be extended to 3-D:

$$W = \exp[Q(\mathbf{E}) - 1], \tag{26.17}$$

where $Q(\mathbf{E})$ is a polynomial function of the strain components whose functional form allows for different degrees of anisotropy.

Classically, constitutive models were phenomenological in nature and not derived from microstructure; however, many current investigators are developing multiscale models that incorporate microstructural composition, fiber–matrix interactions, and fiber orientation to predict both elastic and plastic behavior [6–8]. Such models feature

increasing complexity, and closed-form solutions are often impossible, although modern computing power allows for the solution of the incremental constitutive relations.

Many biological tissues exhibit other characteristics such as time dependence (viscoelasticity) that are essential to their function in vivo and must be incorporated to describe tissue properties accurately. Articular cartilage, for example, exhibits multiphasic behavior in which the matrix composition and interstitial fluid–matrix interactions are essential to conferring the lubricating and impact-absorbing properties of the tissue. Textbooks by Fung and Cowin provide further reading on constitutive modeling of time-dependent tissues [9,10].

Finally, validity of assumptions and predictive capabilities of constitutive models must be evaluated by experiments. Often, simple experiments (e.g., tension, compression, biaxial stretch) can be used to validate the models before application, but the capability of the models to predict essential behavior under near-physiologic conditions is critical.

Boundary Value Problems

Once the tissue properties and behavior are known, determining the actual stresses and strains experienced by cells within the extracellular matrix (ECM) requires solution of the boundary value problems defined by the physical field equations, the constitutive behavior, and the boundary conditions. Boundary conditions are simply the loads and deformations applied further up in the structural hierarchy that induce local stresses and strains at the level of interest. For classical engineering materials, the boundary value problems and admissible assumptions have been identified, and most current research focuses on their solutions; however, in the field of biomechanics, the formulation of boundary value problems, including constitutive models and boundary conditions, remains an active area of research with great promise for enhancing our understanding of biological materials [2].

UNDERSTAND THE ROLE OF MECHANICAL STIMULI

With the necessary tools established and a theoretical framework chosen, we can begin to understand how mechanical stimuli affect organs, tissues, and cells. Mechanical loads are essential for proper morphogenesis (see Chapter 24) and maintenance of normal tissue structure and function in a wide variety of tissues. In many systems, physical stimuli may also be pathogenic, inducing damage or disease, depending on the type, magnitude, and/or frequency of the stimulus. Understanding these factors is therefore important to regenerating mechanosensitive tissues.

In many tissues, subcellular components to whole tissues are coupled to adapt the structure and properties actively according to the mechanical environment. The role of mechanical stimulation has been studied and described at many hierarchical levels, linking gross tissue remodeling to mechanotransduction, the cellular and subcellular responses that convert mechanical stimuli into chemical signals.

Tissue Remodeling

We will first consider bone adaptation as an example of tissue-level remodeling to physical stresses. The mechanisms behind bone tissue remodeling were first described by Frost, who suggested that modeling and remodeling are mediated by basic multicellular units (BMU) made up of osteoblasts, osteoclasts, and osteocytes [11–14]. Osteoblasts lay down bone matrix and osteoclasts degrade it within a highly regulated and intertwining milieu of chemical signals. Osteocytes, residing within bone matrix and communicating with other cells through the lacuno-canalicular network, are thought to be the primary mechanosensors that transduce mechanical signals into chemical signals. These adaptations are stimulated by changes in the mechanical loading history.

Turner and others have studied numerous mechanical variables affecting bone adaptation [15–19] and have proposed three rules for load-induced adaptation [20]. First, bone adapts to dynamic but not static strains. Experimental observations revealed that the strain stimulus, or the strain needed to induce adaptation, was proportional to both strain magnitude and frequency:

$$E = k_1 \varepsilon f, \tag{26.18}$$

where E is the strain stimulus, k is a proportionality constant, ε is the peak-to-peak strain magnitude, and f is the loading frequency [19]. Second is the principle of diminishing returns: that is, as loading duration is increased, the bone formation response tends to level off. This effect was mathematically described by Carter and colleagues as:

$$S = k_2 \left[\sum_{j=1}^{n} N_j \sigma_j^m \right]^{\frac{1}{m}}, \tag{26.19}$$

where k_2 is a constant, N is number of loading cycles per day, σ is the effective stress, and m is a constant weighting factor, which has been estimated at 3.5–4, based on published data [20,21]. Finally, bone adaptation is error-driven such that bone cells accommodate to "normal" strain waveforms but adapt to abnormal strain changes [22]. This has been described mathematically as:

$$\frac{\partial M}{\partial t} = B\{\varphi - F\}, \tag{26.20}$$

where M is bone mass, t is time, φ is the local stress–strain state, and B and F are constants that describe the "normal" load state [23]. Thus, $\varphi - F$ represents the error function driving force for bone mass adaptation.

These rules apply to both mechanical stimulation of new bone formation and disuse-induced bone resorption. Astronauts, for example, experience significant reductions in bone mass: When the local stress–strain state, φ, becomes less than the normal earthbound state, F, because of reduced gravitational loads, the negative error function drives bone resorption. This tightly regulated system can also become pathogenic in osteoporosis, in which the communication between constituents of the BMU is disrupted and more bone is resorbed than can be replaced, leading to a decrease in bone mass and skeletal fragility.

In blood vessels, hemodynamic forces have multiple important roles in regulating vascular cells [24]. Pulsatile intramural pressures produce cyclic strain within vessel wall, and blood flows exert shear stresses on the lumen walls. These two types of physical stimuli influence the phenotype and activity of smooth muscle cells and endothelial cells within the vasculature. Tremendous research has been directed toward studying hemodynamic effects given the potential implications for preventing or treating atherosclerosis, as well as for vascular tissue engineering. Arteries are capable of remodeling their structure in response to changes in their mechanical environment. A chronic increase in systemic blood pressure induces an increase in vessel wall thickness and area, whereas reduced pressure leads to a decrease in vessel dimensions [25].

In cartilage, normal joint loading produces compressive, tensile, and shear forces that deform the cells (chondrocytes) and induce interstitial fluid flows and streaming potentials throughout the matrix [26]. These mechanical, chemical, and electric signals prominently influence the metabolism of the chondrocytes. Because articular cartilage in adults is devoid of a blood supply, mechanical deformations are critically important to facilitate the flow of nutrients and waste products into and out of the tissue. Mechanical deformations also serve to maintain the tissue's proper matrix composition, organization, and mechanical properties. It is generally accepted that static or constant compression and pressure result in loss and/or reduction of synthesis of proteoglycans and DNA in nearly a dose-dependent manner [27]. Dynamic compression has been shown to modulate proteoglycan synthesis positively, and this stimulation is heavily influenced by both the frequency and amplitude of the compressive waveform [27]. Similarly, dynamic tissue shear also has a pronounced effect on matrix components in a frequency- and amplitude-dependent manner [28].

Abnormal joint loads have been shown to induce changes in the composition, structure, and mechanical properties of articular cartilage. For example, disuse studies that employ casting or other means of immobilization have demonstrated a loss of matrix constituents such as proteoglycans and a reduction in tissue thickness and mechanical properties [29]. Conversely, moderate exercise may have beneficial effects on maintaining healthy articular cartilage [30]. Dynamic compression modulates biomarkers implicated in important disease states (e.g., osteoarthritis) such as cartilage oligomeric matrix protein [31], but high-impact loading or altered joint loading resulting from instability or injury is recognized as a significant risk factor for the development and progression of osteoarthritis [30,32]. These studies suggest that there is a range of local stresses and strains that promote healthy tissue homeostasis, but loading conditions that are abnormally high or low can trigger catabolic responses and a loss of tissue function.

Mechanotransduction

How are local mechanical signals transduced into cellular responses that affect tissue growth, repair, and remodeling? The process of mechanotransduction can be divided into four stages [33], as shown in Fig. 26.5: (1) force

transmission, (2) mechanotransduction, (3) signal propagation, and (4) cellular response. The first stage refers to the transmission of the force from the point at which it is applied to the cell surface. The second corresponds to the sensory action of the cells in sensing mechanical stimuli and transducing it into a biochemical signal, which is propagated inside the cell in the third stage. Finally, the cell responds to the intracellular signal by modulating gene expression, completing the mechanotransduction process.

In the first stage of mechanotransduction, applied forces are converted into local stimuli that may be detected by cells. Transmitted forces can cause direct cellular deformation by deforming the surrounding ECM. Applied forces may also result in local fluid flow and/or hydrostatic pressures. For example, compression of articular cartilage generates hydrostatic pressure that can regulate chondrocyte metabolism. Dynamic compression of cartilage induces fluid flow through the matrix and exposes cells to local shear stresses. The relative importance of these different types of local stimuli in vivo is not clear because of the difficulty of isolating each kind of mechanical stimulus. However, extensive research has been done to study the effects of various forms of mechanical stimuli on cells in vitro. These include tensile stretch, compression, hydrostatic pressure, and fluid flow—induced shear stress applied either statically or dynamically. These studies have allowed investigators to identify potential mechanotransduction mechanisms.

The next stage of mechanotransduction occurs at the plasma membrane of the cell, and it is here that the cell detects the external signal and converts it into an intracellular signal. The plasma membrane contains numerous receptors and ion channels that can serve as sensors of the mechanical stimuli. The key structures in this interaction are the mechanosensitive (also known as stretch-activated) ion channels, integrin receptors, and other plasma membrane receptors.

Mechanosensitive ion channels [34—36] are thought to be important to many cell types including chondrocytes [37,38], osteoblasts [39], endothelial cells [40], and cardiac myocytes [41]. Experiments involving direct perturbation of the chondrocyte membrane have implicated such ion channels in the increase in concentration of cytosolic calcium ion [42], which is a second messenger and has well-known intracellular effects [43—45].

Integrins are heterodimeric transmembrane proteins that bind to ECM proteins and cluster together leading to the assembly of focal adhesions, at which the cell contacts the ECM. Focal adhesions associate intracellularly with α-actinin [46], talin [47], tensin [48], and other cytoskeletal binding proteins as well as signaling molecules such as focal adhesion kinase [49]. Because of their associations with both structural and signaling proteins, integrins are well-placed to act as transducers of physical stimuli and have been implicated as a link between the extracellular and intracellular environments for a variety of cell types that allow transmission of inside-out and outside-in signals capable of modulating cell behavior [50—54]. Wright et al. reported that the transduction pathways involved in the hyperpolarization response of human articular chondrocytes in vitro after cyclical pressure-induced strain involve α5β1 integrin, which they suggest is an important chondrocyte mechanoreceptor [54]. Externally applied forces would cause changes in the conformations of the ECM molecules that would affect their binding to integrins and modify the force balance within focal adhesions. It is thought that increased tension within focal adhesions can trigger increased integrin clustering and focal adhesion kinase phosphorylation [55,56], which initiates a signal cascade resulting in altered gene expression.

In addition to integrins, the plasma membrane is host to other receptors for specific ECM proteins such as collagen, aggrecan, and hyaluronic acid, which may also be able to sense extracellular forces because of their interactions with their ligands. G protein—coupled receptors (GPCR) may also act as mechanotransducers or be activated as a result of other pathways, because the consequences of G protein stimulation of the phospholipase C—inositol triphosphate (IP_3) pathway have been observed in mechanically stimulated cells [40,57].

Primary cilia, microtubule-based, flagella-like extensions of the membrane, have been identified as potent mechanosensors [58]. First identified in the late 1800s and thought to be a functionless vestige, the primary cilium has been implicated as a mechanism for mechanosensation in numerous cell types including kidney [59], bone [60,61], and cartilage [62]. Jacobs and colleagues identified primary cilia in both osteocytes and osteoblasts and proposed that primary cilia sense lacuno-canalicular fluid flow caused by bone loading [61]. They demonstrated that primary cilia are required for osteocyte and osteoblast response to fluid shear stress, inducing the expression of genes (osteopontin, cyclooxygenase-2, and osteoprotegerin/receptor activator of nuclear factor κB ligand) and production of second messengers (prostaglandin E_2 [PGE_2]) associated with bone remodeling. Primary cilia have also been shown to cause intracellular Ca^{2+} release by GPCR proteolysis of polycystic kidney disease 1 to activate Runt-related transcription factor 2 (Runx2) and IP_3 production [63]. Further research is required to fully understand the mechanisms through which primary cilia respond to mechanical stimuli.

The third stage of mechanotransduction is signal propagation, in which extracellular physical stimuli propagate into the cell through cytoskeletal remodeling, calcium flux, and kinase cascade activation leading to midterm and

long-term changes in transcriptional activity [64]. A complex and incompletely understood transcription factor-mediated integration system is responsible for much cellular adaptation to physical environmental cues, regulating tissue morphogenesis. Coordinated activation and/or expression of multiple transcription factors combinatorially regulate downstream expression patterns, forming a signal integration system that interprets the cells' physical, chemical, and microenvironmental context and returns the necessary response [64].

Advances in transcriptomics have led to the identification of novel transcriptional activators whose transcriptional targets overlap with known mechanotransducers. These include nuclear factor-κB (NF-κB), serum response factor, as well as Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding domain (TAZ) [65]. YAP and TAZ have received extensive attention because they appear to be nearly ubiquitous effectors of mechanosensation. These proteins are paralogous transcriptional coactivators that translocate to the nucleus to activate a number of transcription factors, most notably the TEA domain family (TEAD1−4) [66,67]. YAP and TAZ are localized to the nucleus on rigid substrates in response to dynamic loading, and require Rho GTPases (RHOA, CDC42, and RAC1) in addition to a number of actin processing factors [68−70]. YAP and TAZ nuclear localization is inhibited by cell−cell contact-mediated activation of hippo core kinases mammalian STE20-like protein kinase and large tumor suppressor kinase 1; subsequent serine (S127YAP/S89TAZ) phosphorylation, nuclear restriction, and degradation of YAP and TAZ [65].

Differentiation typically requires exit from a proliferative progenitor state to a functional, nonproliferative state, and can be directly controlled by both dynamic and passive mechanical cues [71]. For example, differentiation of pluripotent stem cells to motor neurons requires loss of homeobox transcription factor nanog, SRY-box 2 (SOX2), and octamer binding transcription factor 4 and induction of SOX1 and Paired box 6 and is accelerated by highly compliant substrates that promote phosphorylation and deactivation of YAP/TAZ- and mothers against decapentaplegic (Mad) and the *Caenorhabditis elegans* protein Sma (SMAD)-mediated transcription [72]. Dynamic strain also has a key role in morphogenesis; in the case of cartilage, compressive loads promote chondrocyte differentiation and matrix production in developing limb buds through cyclic adenosine monophosphate (cAMP) activation of cAMP-dependent protein kinase A and subsequent phosphorylation and association of cAMP response element binding protein and SOX9 [73]. However, context is important because, for example, cyclic stretch has been shown to have both pro- and antimyogenic capacity in C2C12 myoblasts through activation of NF-κB−mediated gene expression [74]. Finally, flow and shear force have a role in diverse cellular function. This is most evident in the cardiovascular system. Regions in the vascular tree are less susceptible to atherosclerotic lesions and inflammation as a result of atheroprotective shear flow [75]. Shear forces activate extracellular signal-regulated kinases and promote myocyte enhancer factor 2 and NF-κB nuclear localization and Krüppel-like factor 2 expression, which promote atheroprotective antiinflammatory gene expression, most notably nitric oxide synthase (NOS) [75,76]. Shear flow−generated NOS produces nitric oxide (NO), a potent vasodilator required for initial hematopoietic stem cell emergence during embryogenesis, which means that cardiovascular flow is required for hematopoiesis [77].

Similarly, the β-catenin pathway has sparked great interest as a mechanotransduction mechanism. Like YAP and TAZ, intracellular β-catenin is normally controlled by subcellular localization and proteasomal degradation in the cytosol [78]. Under mechanical stimulation, cells produce small molecules known as Wnts that bind to the membrane receptor complex of LRP5/6 and Frizzled to phosphorylate the kinase glycogen synthase kinase-3β, resulting in deactivation of the destruction complex [79,80]. This allows stabilization of intracellular β-catenin, which translocates to the nucleus to initiate gene expression, and in osteoblasts for example, it induces bone formation [81,82]. Interestingly, mechanical stimulation of osteocytes has also been demonstrated to activate the β-catenin pathway independently of Wnt signaling through NO and phosphatidyl inositol-3 kinase [83]. This pathway has the potential to provide novel targets for intervention in bone-remodeling pathologies and to manipulate the response of cells to mechanical stimuli.

Mechanotransduction is an essential component of a number of pathologic and regenerative processes: fibrosis, differentiation, and wound repair. Injury-induced transforming growth factor-β (TGF-β)-mediated SMAD signaling promotes an inflammatory response that coordinates with YAP and TAZ signaling to promote proliferation and tissue remodeling [84,85]. However, during fibrosis and cancer-associated fibroblast activation, pathologic ECM stiffening increases YAP and TAZ signaling that can overcome contact inhibition and result in an excessive wound healing response [86,87]. Likewise, bone marrow stromal cell tripotentiality is coordinated by wingless-type MMTV integration site modulation of β-catenin that is inhibited by YAP and TAZ through direct or indirect action on the β-catenin destruction complex [69,88]. Osteogenesis in particular requires rigidity-mediated nuclear localization of TAZ to enhance Runx2 activity, and YAP and TAZ together may have a long-term role in bone marrow stem cell lineage restriction [89−91].

The final stage of mechanotransduction is the altered response of the cell, which may include changes in matrix synthesis and degradation, proliferation, differentiation, apoptosis, cell alignment, and migration. The effectors of the mechanotransduction pathways are the various transcription factors, which are activated by the events discussed previously. The activated transcription factors interact with the promoter and enhancer regions of various genes to mediate transcription. This results in an increase in the expression of genes, such as in vascular cells, Cox-2, vascular endothelial growth factor, TGF-β3, and endothelial NOS (eNOS) [92], which orchestrate the cellular responses. Lee et al. demonstrated that vascular smooth muscle cells respond to mechanical strain by increasing specific proteoglycan synthesis and aggregation [93]. It is known that mechanical loading of osteocytes results in anabolic responses such as the expression of c-fos, insulin-like growth factor-I, and osteocalcin [94]. Elevations in Ca^{2+} activate a Ca^{2+}/calmodulin-dependent protein kinase that causes increased c-fos expression, which is a pro-growth transcription factor. Calcineurin, a Ca^{2+}/calmodulin-activated phosphatase, dephosphorylates and activates the nuclear factor of activated T cells (NF-AT) family of transcription factors. Different NF-ATs expressed in different cells, including those of the heart, cartilage, and bone, serve as tissue-specific activators of cell growth and differentiation [95,96].

MECHANICAL REGULATION OF VASCULARIZED TISSUE REGENERATION

Replacing tissues that serve a significant biomechanical function has proven exceptionally challenging [97]. For mechanosensitive cells and tissues, it may be possible to manipulate the mechanical environment, either in vivo or in a bioreactor, to enhance the integration, degradation, or activity of a tissue-engineered construct.

Mechanical Stimulation In Vivo

During development, bone formation is controlled by the spatiotemporal distribution of morphogens such as TGF-β1 and bone morphogenetic protein-2 (BMP-2). Therefore, presentation of these and other growth factors has been pursued to stimulate regeneration in challenging clinical cases such as large bone defects [98]. For further detail on this topic, please refer to Chapter 24. Notably, however, bone development is also acutely responsive to mechanical stimuli induced by fetal movement. This in utero motion functions to direct joint morphogenesis [99] and promote endochondral ossification [100]. Similarly, in natural fracture healing, the local mechanical environment determines both the course and success of healing [101]. Although it was once held that complete immobilization was needed for successful fracture healing and that the resorptive effect of disuse was necessary to release calcium for callus mineralization [102], it is now known that limited physical activity can promote tissue repair and the restoration of function [103].

Despite strong evidence that the local mechanical environment acutely influences bone healing, studies evaluating the effects of in vivo stresses on engineered tissue regeneration have emerged. Case et al. investigated the effects of controlled intermittent compressive deformation on cellular constructs using a hydraulic bone chamber device implanted into the distal femoral metaphyses of rabbits [104]. Constructs receiving 4 weeks of daily mechanical loading at 0.5 Hz were found to have ninefold more new bone formation compared with contralateral control constructs that did not receive loading. Using preclinical models of large bone defect regeneration, several groups have begun to evaluate the influence of in vivo mechanical loading on tissue-engineered bone repair [105–110]. For example, Boerckel et al. developed a rat segmental bone defect model that enables the control of ambulatory load transfer through modulation of axial fixation plate stiffness [106] (Fig. 26.4A). These studies demonstrated that bone regeneration depends on both the magnitude and timing of in vivo loading, with early loading inhibiting vascular ingrowth leading to pseudarthrosis but delayed loading enhancing bone regeneration and remodeling leading to restored functional bone properties [105–107] (Fig. 26.4B,C). Notably, this delayed loading protocol failed to induce regeneration in the presence of structural scaffolds that mimicked the properties of native bone [110], which suggests that stiff scaffolds can impede the beneficial effect of mechanical loading by stress shielding or inhibition of tissue ingrowth. Collectively, these observations suggest that the mechanical environment should be considered together with the physical and biochemical properties of engineered extracellular matrices for tissue regeneration.

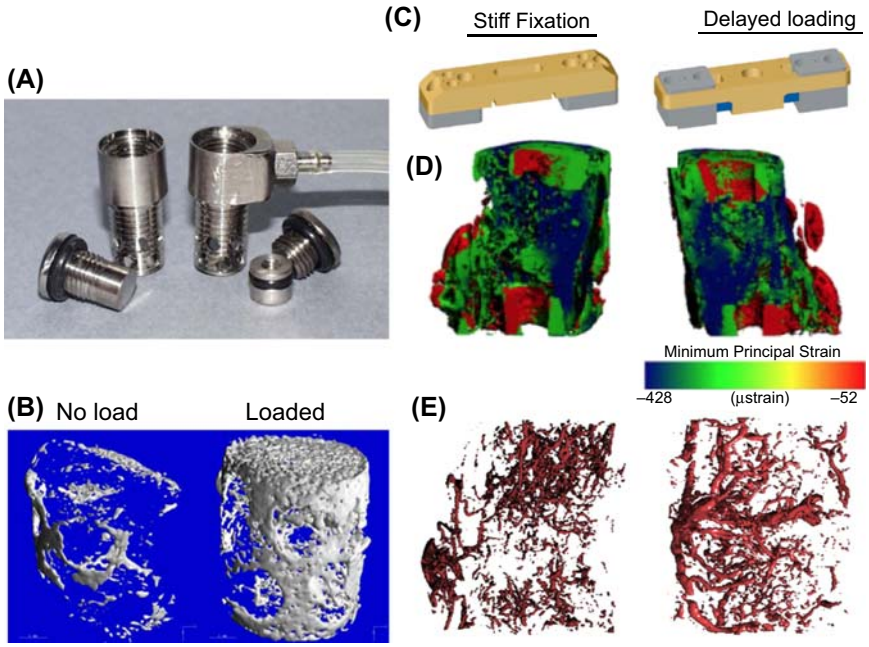

FIGURE 26.4 (A) Hydraulic bone chamber implant used to apply cyclic compressive loading to tissue engineered constructs in vivo. Implanted constructs (B) receiving the mechanical stimulus (right) had 9-fold more new bone formation than no load controls (left). (C) Stiff (top) and axially compliant (below) fixation plates. (D) Compliant fixation (right) enhanced bone formation and altered local strain disributions over stiff fixation (left). (E) Delayed loading increased vascularization compared to stiff fixation.

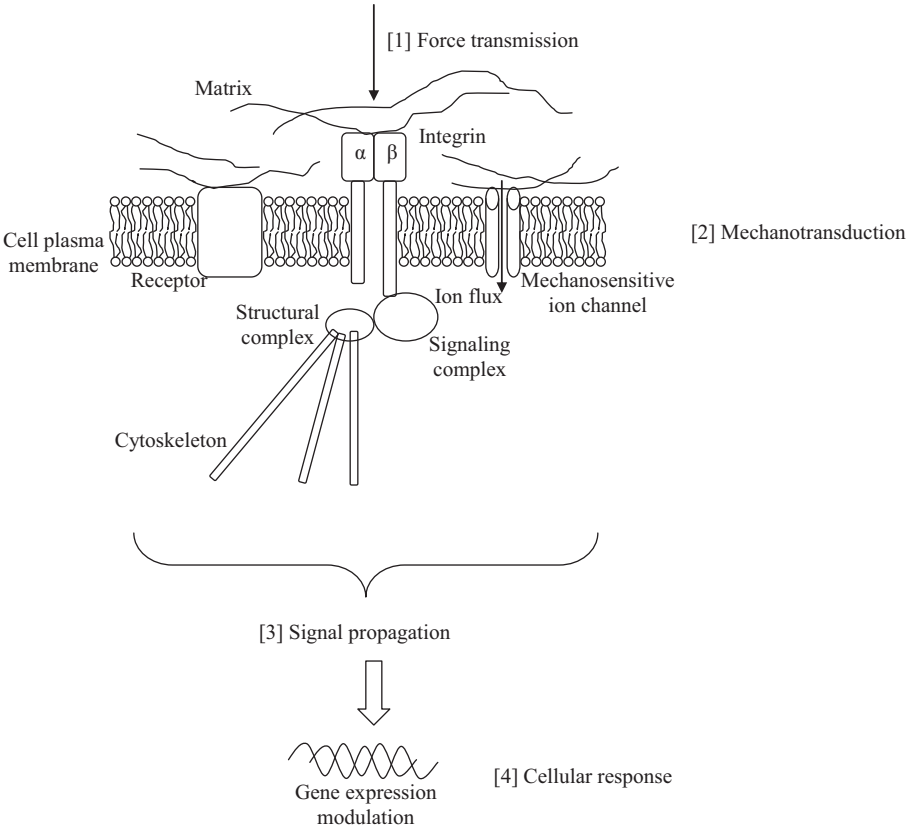

FIGURE 26.5 Schematic showing the four stages of mechanotransduction: [1] force transmission, [2] mechanotransduction, [3] signal propagation, and [4] cellular response. See text for details.

Mechanical Stimulation In Vitro

Many tissues bear tremendous stress and strain over repeated loading cycles in vivo while maintaining normal function. To date, no engineered construct has been developed in vitro possessing the same biomechanical properties as its in situ counterpart. One approach to addressing this challenge is to use physiologically inspired mechanical forces to transmit stimuli to developing constructs in vitro. Because these tissues normally experience a dynamic environment in vivo, the rationale is that the application of mechanical forces such as compression or shear stress will stimulate the cells of the engineered construct to secrete and organize the proper matrix proteins required to reproduce the mechanical function of the native tissue.

Perhaps the tissues of the body most subjected to mechanical forces are those of the musculoskeletal and cardiovascular origin. Consequently, orthopedic and cardiovascular tissue-engineered constructs represent the bulk of the research in which mechanical forces have been applied to developing tissues in vitro. Cartilage, bone, tendon, ligament, blood vessels, heart valves, and muscle have been cultured in vitro under the influence of mechanical forces. The remainder of this section will discuss select examples from the orthopedic and cardiovascular fields that use the in vivo environment as an inspiration to mechanically condition tissue-engineered constructs in vitro.

Cartilage Bioreactors

Cartilage bioreactors commonly apply compression and/or shear forces to modulate the construct matrix composition and mechanical properties. Although many different tissue-engineering models exist for cartilage (e.g., alginate, agarose, pellet/micromass, and scaffold and scaffold-free culture), the mechanical properties necessary to withstand the complex and demanding in vivo mechanical environment have yet to be recapitulated. For clinical success, it has been suggested that tissue-engineered constructs may need to approximate the matrix composition, organization, and biomechanical properties of native tissue to promote construct integration and load-bearing capability in vivo [111]. Bioreactor systems have produced encouraging results indicating that in vitro mechanical conditioning of tissue-engineered constructs is a promising approach to reproducing native tissue properties.

As one example, a novel dual-chambered, parallel-plate flow bioreactor system has been used to apply controlled shear stresses to the surface of cartilaginous constructs grown de novo from primary bovine articular chondrocytes without the aid of a scaffold (Fig. 26.6). The "parallel-plate" design refers to the top bioreactor surface and tissue-engineered construct face that form two parallel walls separated by a defined distance that creates a flow channel. Fluid flows through the channel, resulting in a parabolic velocity profile. Consequently, a shear stress is applied that is maximal at the upper wall and tissue surface; this is commonly referred to as Poiseuille flow [112]. One can estimate the wall shear stress (τ_w) by the following equation:

$$\tau_w = \frac{6\mu Q}{bh^2}. \tag{26.21}$$

where μ is the media viscosity, Q is the volumetric flow rate, b is the flow chamber width, and h is the fluid gap height.

Chondrocytes are seeded onto a semipermeable membrane; following a static preculture period, fluid-induced shear stress is applied to the construct. The application of flow significantly increases type II collagen compared with static (no flow) controls, as well as both Young's modulus and ultimate strength [113]. This study suggests that flow-induced shear stresses may be an effective functional tissue-engineering strategy for modulating matrix composition and mechanical properties in vitro.

Bone Bioreactors

Without a vascular blood supply in vitro, nutrient delivery to cells throughout 3-D tissue-engineered constructs grown in static culture must occur by simple diffusion alone. As a result, attempts to engineer bone greater than 1 mm thick usually result in a thin shell of viable tissue and cells localized at the periphery [114]. It has been theorized that this effect results from suboptimal mass transport conditions and a lack of mechanical stimulation in static culture. Therefore, tissue culture systems that provide dynamic media flow around or within tissue-engineered constructs have been designed to enhance nutrient and waste exchange in vitro [115]. In addition to enhancing mass transport, fluid flow applies shear stresses to the cells within the scaffolds. The effects of flow-mediated shear on cells have been studied in 2-D monolayer cultures. Continuous fluid flow applied to osteoblasts in vitro has been shown to alter bone-related gene expression and the cellular phenotype [116]. Parallel-plate flow experiments have shown that bone cells cultured in monolayer are highly responsive to flow-mediated shear stresses. Shear stresses in the range of 5–15 dyn/cm^2 affect osteoblast proliferation as well as the production of NO and PGE_2,

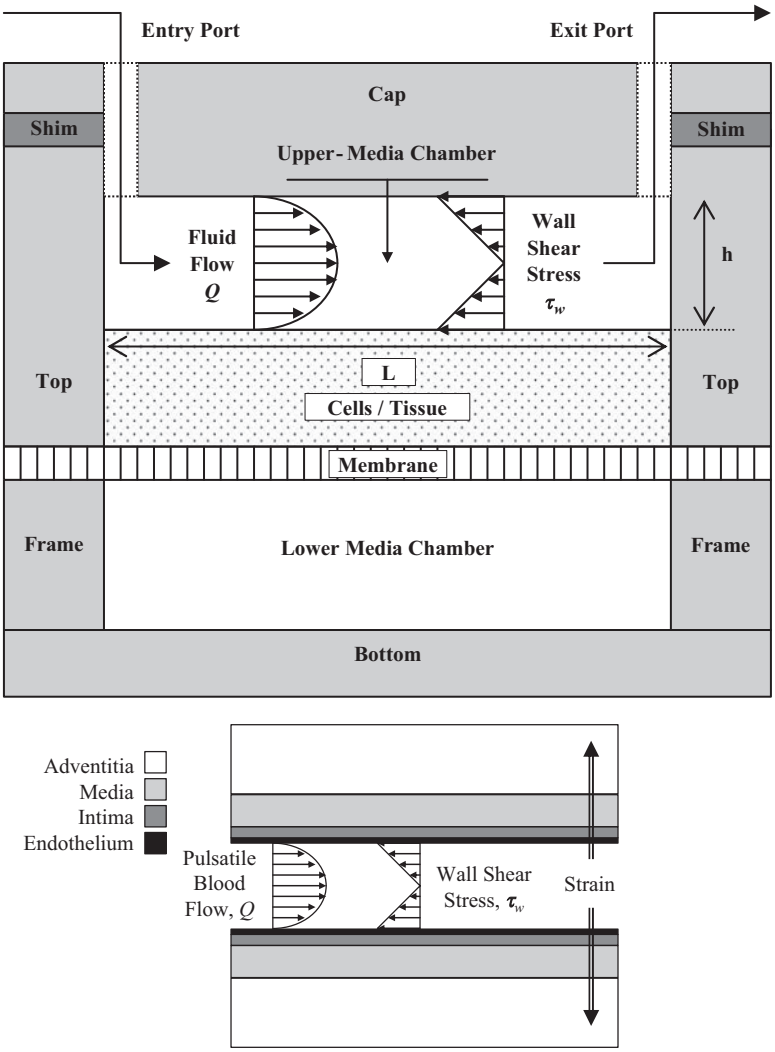

FIGURE 26.6 Dual-chambered parallel-plate bioreactor system that applies controlled shear stresses to the surface of cartilaginous construct slabs.

which suggests that shear stress is an important regulator of osteoblast function [117]. Pulsatile and oscillatory flow conditions applied to osteoblasts using in vitro parallel-plate flow chambers have also been shown to increase gene expression, intracellular calcium concentration, and the production of NO and PGE_2 compared with static controls [118,119]. Furthermore, cell responsiveness has been reported to vary with the fluid flow rate and frequency [120,121]. Proposed mechanisms for the stimulation of cells by fluid flow include increased mass transport, the generation of streaming potentials, and the application of shear stresses to the cell membranes [119,122]. Although these studies were performed using 2-D cell culture systems for short-term experiments, they suggest that variable flow conditions may also have differential effects in 3-D tissue culture systems.

Such tissue culture systems may be useful to engineer thicker, more uniform bone graft substitutes for implantation or as test bed models that simulate aspects of the in vivo environment. Whereas many different bioreactor systems have been developed, perfusion bioreactors in particular have shown significant increases in both cell viability and mineralized matrix formation on large 3-D constructs in vitro. In a study, micro—computed tomography has been used to quantify mineralized matrix production within perfused and statically cultured marrow progenitor cells seeded on large polymer scaffolds (6.35 mm in diameter and 9 mm thick) [123]. Statically cultured constructs were found to have mineralized matrix localized only to the periphery of the constructs. In contrast, perfused constructs were found to have a several-fold increase in mineralized matrix production distributed throughout the constructs (Fig. 26.7).

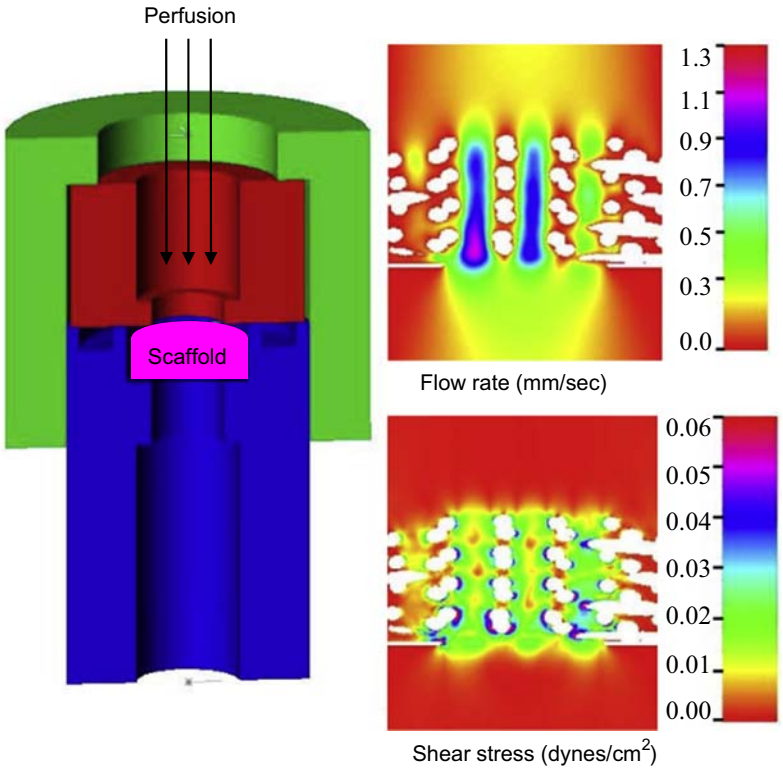

FIGURE 26.7 Perfusion bioreactor system (left) for production of mineralized constructs for bone defects. Computational fluid dynamics simulation of flow rate and shear stresses within the 3D scaffold porosity (right).

Blood Vessel Bioreactors

Following the same rationale for mechanical conditioning of orthopedic-engineered tissues, cardiovascular tissues can also be enhanced by in vitro mechanical stimulation. Cardiovascular tissues reside in a dynamic environment that can be mimicked in vitro using bioreactors and mechanical loading systems to deliver the physiologically inspired environmental cues.

In vivo, the pulsatile flow of blood imparts cyclic strains and shear stresses to the vessel's constituents, which respond in a variety of ways to these mechanical signals. Endothelial cells are uniquely situated in the lumen and are directly in contact with the flowing blood, which causes a shear stress to be applied to the cells. Consequently, these rapidly responding, mechanosensitive cells attain an elongated shape, aligning their long axis with the direction of flow. Sensing of the shear via cell-surface receptors, ion channels, or integrins leads to the secretion and/or activation of a number of signaling molecules such as NO, eNOS, kinases, and transcription factors [124–126]. Perhaps most important, the fluid-induced shear stress confers a protective effect on the vessel by decreasing the probability of atherosclerosis [127]. Indeed, areas of irregular blood flow (i.e., velocity, direction, and shear stress) have been implicated as sites of increased atherosclerosis [128]. Shear stress also modulates smooth muscle cell production of signaling molecules (such as NO) [129] and gene transcription levels of cell-surface receptors [130].

Tissue-engineered blood vessels (TEBVs) aim to reproduce cellular and mechanical properties of the native vessel as an effective replacement. However, similar to other engineered tissues, those cultured under static conditions fall short of native tissue properties. The concept of mechanical stimulation of TEBVs to enhance matrix organization and mechanical properties began in the mid-1990s, and it has since been an active topic of research. Historically, three types of TEBVs have received the greatest attention for application of in vitro cyclic strains. First, collagen gel-derived TEBVs were exposed to cyclic circumferential strains by Nerem and Tranquillo and colleagues. Cummings et al. demonstrated an increase in mechanical properties and altered cellular function in response to cyclic mechanical strain of smooth muscle cell–seeded collagen I matrices. Likewise, Seliktar et al. showed that 10% cyclic strain of cell-seeded collagen gels induced matrix metalloproteinase 2 (MMP-2)–mediated remodeling, yielding improved mechanical properties [131]. Gleason and colleagues presented a microstructurally motivated continuum mechanics model that combines numerous factors affecting the success of the TEBV approach, such as cell type,

matrix composition, mechanical stimulus, and their interacting effects on TEBV growth, remodeling, and mechanics [132]. Such studies point to the challenge of optimizing this approach and point to the importance of biomechanics-informed rational experimental design.

Second, the effects of cyclic stretching on biodegradable polymeric scaffolds have been investigated. Gong and Niklason reported that cyclic strain of cell-seeded constructs enhanced mesenchymal stem cell differentiation toward a smooth muscle cell phenotype and induced a more normal tissue composition [133].

Finally, Auger and colleagues presented a method of self-assembly, in which cells are cultured in two dimensions to excrete their own ECM, forming a tissue sheet, which is then rolled into a TEBV. When exposed to uniaxial stretch, the cells realign along the axis of strain and improve the contractile capacity of the resulting TEBVs [134].

Other experiments demonstrated that exposing tissue-engineered vascular grafts to fluid-induced shear stress increased endothelial cell adherence [135] and proliferation [136] and altered tissue morphology and mechanical properties [137]. Cyclic mechanical strains cause an increase in collagen (types I and III) transcription by smooth muscle cells [138], an increase in mechanical properties (strength and stiffness) that is attributed to an increase in remodeling enzymes such as MMP-2 [139] and an increase in matrix and cellular organization [136,140]. Subjecting smooth muscle cell−impregnated constructs to dynamic mechanical stress not only causes ultrastructural and orientation changes in the cell phenotype and matrix, it can also induce cells to shift from a synthetic to a contractile state [141]. Similar constructs (smooth muscle cells seeded into polyglycolic acid meshes) exposed to pulsatile radial stresses of 165 beats per minute (analogous to fetal heart rates) and 5% radial strain produces constructs with burst pressures in excess of 2000 mm Hg increased collagen deposition and desirable histological characteristics [142].

Great strides have been made in the field of tissue-engineered vascular grafts, but a completely successful graft has yet to be identified. However, as the field continues to progress and learn more about the in vivo environment, those cues can be translated to more realistic conditioning techniques for in vitro−grown constructs. This mechanical stimulation is critical to remodeling the graft to possess proper mechanical properties as well as matrix composition and organization. The same can be said for cartilage and bone. Thus, mechanical conditioning in an in vitro setting has proven to be a powerful technique to increase the similarity of tissue-engineered constructs to the native tissues they aim to replace.

EVALUATE FUNCTIONAL RESTORATION

Assessment of functional regeneration is an essential benchmark for establishing a successful tissue-engineering strategy. Often, qualitative, indirect measures of regeneration are presented without evaluating biomechanical integrity. For tissues and structures whose primary function is to bear physical loads, mechanical testing is an essential measure of repair. In cartilage regeneration, for example, many studies present only compositional and morphological assessments of healing without directly measuring mechanical function. Although composition and morphology are important measures, the true indicator of regeneration is whether the regenerated tissue recapitulates normal tissue behavior, including both the monotonic elastic and viscoelastic properties. Although it is often experimentally difficult, the establishment of standards for mechanical evaluation of engineered tissues will be a significant contribution. For tissue-engineering approaches to long bone defect healing, torsion testing is an experimentally facile and analytically simple method of determining the structural properties of the regenerated tissue and allows for direct comparison with age-matched uninjured limbs.

Other functional considerations include the assessment of long-term consequences and temporal remodeling of tissue-engineered constructs. These include the evaluation of scaffold degradation by-products, the extent of remodeling to native architecture, and the restoration of the behavior of the native material.

CONCLUSIONS

This chapter presented four steps to approaching a tissue-engineering problem successfully from the perspective of physical mechanics: (1) describe the native mechanical environment quantitatively, (2) understand the role of mechanical factors in the tissue of interest, (3) manipulate mechanical conditions to enhance function or regeneration, and (4) evaluate the degree of functional biomechanical restoration of the engineered tissue quantitatively. Although all of these considerations are active areas of research, regenerating tissues that serve a significant biomechanical function continue to prove exceptionally challenging [97]. It is clear that tissue regeneration strategies must take

into consideration the complex and demanding in vivo mechanical environment into which tissue-engineered constructs are implanted. Fortunately, a wealth of knowledge is available to tissue engineers regarding how local stresses and strains affect cell function within tissues. Integration of this knowledge into strategies for tissue replacement or regeneration will be important to achieving the goal of long-term functional restoration in patients.

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CHAPTER

27

Cell–Substrate Interactions

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INTRODUCTION

In most cases, tissue engineering in vitro or in vivo requires a scaffold to provide the optimal microenvironment for seeded cells. There is a growing trend toward using synthetic substrates to mimic natural, physiological systems for tissue engineering or regenerative medicine. Cell–substrate interactions are fundamentally important to studies geared toward designing biomimetic substrates that may replace damaged vital organs or tissues or assist in the natural healing processes of the body. This chapter reviews cell interactions with the extracellular matrix (ECM). This information is followed by sections detailing the modification of cell behavior by different aspects of a biomimetic substrate, such as its physical, chemical, and biological properties. The role of surface topography in modulating cell interactions is also discussed. Many studies have underscored the necessity of a three-dimensional (3D) environment to yield physiologically relevant data; thus, a section is dedicated to the effect of dimensionality on cell behavior. The chapter concludes with a discussion on the importance of mechanical stress in tissue development at both the cellular and tissue levels.

CELL–EXTRACELLULAR MATRIX INTERACTIONS

The principal building blocks of organs are cells. These functional units are held together to become structures such as tissues, by a hydrated gel-like material known as the ECM. The ECM provides spatial organization, anchorage, and mechanical strength for different cells within a tissue. In addition, it is also responsible for the control and regulation of cell functions such as adhesion, spreading, proliferation, migration, differentiation, and apoptosis by providing mechanical as well as biochemical stimuli.

The functions of the ECM are carried out by important components such as fibrillar and nonfibrillar glycoproteins, hydrated proteoglycans (insoluble macromolecules), and soluble molecules such as growth factors and cytokines [1]. The most important fibrous proteins that cross-link the matrix are collagen and elastin, which contribute to the tensile and contractile strength of the tissue, respectively. For instance, elastin creates the recoil after transient stretch [2] such as contraction of the heart tissue. Nonfibrous proteins include fibronectin (FN), vitronectin, and laminin, which act as anchors that initiate cell binding via cell surface adhesion receptors such as integrins. These proteins also stimulate cell signaling pathways in a bidirectional manner between the cells and the ECM. Whereas FN is ubiquitous, vitronectin has a greater role in adhesion involving endothelial cells. Laminin is a vital protein secreted by epithelial cells and forms an important constituent of the basal lamina [3].

The integrin family of cell adhesion receptors consists of heterodimeric glycoproteins (composed of α and β subunits) that show specificity for different cell adhesion proteins [4] as well as collagen. For example, $\alpha_1\beta_1$ binds collagen whereas $\alpha_5\beta_1$ and $\alpha_v\beta_3$ have affinity specifically for FN and vitronectin. The complete integrin receptor is composed of an extracellular domain that binds the ECM in a cation-dependent manner and short cytoplasmic tails that lack intrinsic kinase activity [5–10]. These proteins mediate cell adhesion via cell surface receptor–ligand

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binding, leading to the clustering of the integrin receptors and formation of transient cell–ECM contacts known as focal contacts. Clustering (or mechanical force and the presence of growth factors) can bring the cytoplasmic segments of the integrins into close proximity, possibly resulting in dimerization or autophosphorylation of tyrosine kinase proteins such as focal adhesion (FA) kinase (FAK).

Focal contacts stabilize into FAs, which are molecularly complex structures containing proteins including α-actinin and talin (which connect integrins to the actin cytoskeleton), paxillin, and signaling molecules such as FAK (bound to integrins directly, but not to the cytoskeleton). Furthermore, FAs contain vital molecules such as vinculin, which link adhesion molecules to actin and other adaptor molecules. All of these molecules transmit signals for various regulatory pathways between the cell and the ECM [11]. These complexes also serve as the termination points of actin filament bundles known as stress fibers. Recruitment of Src homology 2 domain–containing proteins such as Src kinase and p130 by phosphorylated FAK to these complexes may induce their subsequent phosphorylation. In turn, the phosphorylation activates downstream pathways and alters gene expression that is ultimately translated into specific processes such as migration, proliferation, and differentiation. For instance, activation of small guanosine triphosphatase (GTPase) Rac or Erk and c-Jun N-terminal kinase pathways results in cell migration and proliferation, respectively [8].

Glycosaminoglycans such as heparan sulfate, hyaluronic acid, and chondroitin sulfate in the glycosylated proteins are also involved in cell adhesion, cell signaling, and communication. In addition, growth factors and cytokines such as interleukin-2, transforming growth factor β, and platelet-derived growth factor (PDGF), which may be either immobilized or solubilized in the ECM, are responsible for cell proliferation and differentiation. Cell interaction with the ECM has been demonstrated in the adhesion of cells to substrates coated with ECM molecules, a change of cell shape, the migration of cells along a concentration gradient of ECM ligands, and the demonstration of differentiated phenotype (development of neurites) in response to the ECM [12].

CELL–SUBSTRATE INTERACTIONS

Importance of Substrate

Although it may appear that interactions between the ECM and the cells control only cellular functions, in reality ECM–cell communication is bidirectional. The cells can regulate how much ECM is synthesized or the extent to which it is degraded by controlling the amount of ECM degrading the enzymes produced (proteases such as matrix metalloproteases, collagenase, or plasmin). This is an integral part of ECM remodeling. Furthermore, the extent and specific function of the ECM vary from tissue to tissue and are governed by the need and function of the tissue itself (e.g., connective tissue versus epithelial cells). Because the cells are constantly in close contact with the ECM, these two components exert a considerable degree of influence over each other, known as "ECM–cell dynamic reciprocity" [1]. The realm of tissue engineering ranges from basic studies elucidating the mechanisms underlying cell behavior to applications generating tissues and replacing organs. This involves the use of biomaterials that can mimic the natural environment spatially and temporally so as to facilitate cell adhesion, proliferation, or differentiation according to tissue-specific requirements. Within the context of cell–ECM interactions, important design considerations for a potential biomaterial are discussed subsequently. The influence of these design parameters on cell behavior is further illustrated with pertinent examples from the literature.

Effect of Physical Properties

Although metals and ceramics are important biomaterials, polymers are much more commonly used in tissue engineering and regenerative medicine applications because of their versatility. The focus of this chapter is therefore on biomedical polymers. Understanding cell interactions with polymers is important for designing substrata for in vitro cell culture or in vivo implantation. Because cells constantly interact with the extracellular environment, they are sensitive to changes in the surface and bulk properties. In addition, for in vivo application, the mechanical property of the substrate or scaffold may need to match the mechanical requirement of the implantation site so as not to affect the biomechanical stimulus adversely that is provided to the seeded cells in situ. Physiochemical properties such as crystallinity, morphology, and stiffness or compliance of materials affect cell attachment and cellular behavior.

Crystallinity

The crystallinity of a polymer represents a state in which a periodic and repeating atomic arrangement is achieved by molecular chain alignment [13]. Because of their size and complexity, polymer molecules are often only partially crystalline, with crystalline regions dispersed within the amorphous regions. The degree of crystallinity of a polymer depends on the rate of cooling during solidification as well as on chain configuration. Upon cooling through the melting temperature, sufficient time must be allowed for the polymer chains to self-organize and form a crystalline region. Crystallization is favored in polymers with a chemically simple structure. Some common crystalline polymers used in biomedical applications include polyethylene, polypropylene, polytetrafluoroethylene, poly(vinyl chloride), poly(glycolide) (PGA), poly(L-lactide) (PLLA), and poly(caprolactone) (PCL). Crystalline polymers are usually stronger and more resistant to solvent penetration and softening by heat.

Crystallinity affects mechanical properties such as the strength and fatigue resistance of the polymer, but it also has an important role in determining surface physiochemical properties including surface free energy, chemical states, polarity, surface roughness, and wettability, which influence cellular response. When blood compatibility was tested on polypropylene surfaces with different crystalline states, an increase in platelet adhesiveness was observed with decreasing surface crystallinity and interlamellar spacing [14]. A decrease in interlamellar spacing resulted in enhanced albumin adsorption and diminished fibrinogen adsorption. Therefore, controlling the crystalline–amorphous microstructure at the surface layer may improve the blood compatibility of polypropylene surfaces.

When designing scaffolds for implantation, crystallinity can influence the biodegradability and cellular responses of the scaffold. The crystalline region is more resistant to water penetration and hence retards biodegradation. The adhesion, proliferation, and morphology of human articular cartilage chondrocytes were different when cultured on various degradable polymers with various crystallinity, such as PGA, PLLA, poly(D,L-lactide), different ratios of poly(D,L-lactide-*co*-glycolide)s, PCL, poly(glycolide-*co*-trimethylene carbonate) (PCTMC), and poly(dioxanone) films [15]. Significantly higher number of chondrocytes were attached to PGA and 67:33 PCTMC polymer films than on PCL and PLLA films. The total cell numbers and hence fold expansion were significantly higher than the fold expansion on tissue culture polystyrene (TCPS), although the greater fold expansion may be attributed to the lower initial cell attachment. Park and Griffith performed a study of spheroid formation by hepatocytes and proliferation of fibroblasts on PLLA substrates [16]. Their results suggested that cells proliferate more slowly on crystalline versus amorphous PLLA and faster spheroid formation on crystalline substrates. This highlights the interesting dynamics between cell–substrate and cell–cell interactions in dictating cell aggregation. Mikos et al. investigated tissue in growth through porous scaffolds composed of semicrystalline or amorphous PLLA that were implanted in rat mesentery [17]. There was a twofold reduction in percent tissue ingrowth through the crystalline scaffolds after 10 days compared with the amorphous scaffolds.

Variations in crystallinity can also lead to changes in surface roughness on nanometer-length scales [18]. MC3T3-E1 osteoblastic cells showed a higher rate of proliferation on smooth regions with a monotonic variation in rate as a function of roughness.

Morphology

The morphology of the substrate can affect cell attachment by influencing the ability of the substratum to adsorb protein and/or by altering the conformation of the adsorbed protein. ECM proteins are present in serum that is used in most cell cultures. Cell attachment to the substratum is almost always mediated by these ECM proteins adsorbed on the culture surface. Rough and porous surfaces are routinely used in clinical applications such as orthopedic, dental, and cardiovascular prostheses [19–22]. For example, numerous studies suggested that implants with a porous surface can form better tissue–implant seals to enhance tissue integration [21]. Roughness has been shown to alter the adhesiveness of platelets to hydrophobic and hydrophilic surfaces [23]. The details of surface topography and surface chemistry will be discussed in later sections.

Stiffness and Compliance

The stiffness of a material is measured by the modulus of elasticity or Young's modulus; compliance is the inverse of stiffness. Sufficient substrate stiffness is important for anchorage-dependent cells, which often rely on finite resistance to cell-generated forces to induce outside-in mechanical signals. Such signals feed back into cell tension [24], cell adhesion [25], protein expression and cytoskeletal organization [26], and cell viability [27]. Stiffness and compliance encountered during cell–cell adhesion and cell–substrate adhesion are important interactions that modulate intracellular signaling pathways and cellular events from gene expression to cell locomotion.

When Normal Rat Kidney (NRK) epithelial cells and National Institutes of Health (NIH) 3T3 fibroblasts were cultured on collagen I substrates with varying Young's modulus, they exhibited different motility and cytoskeletal organization [28]. Both cells were well-spread on rigid substrates. NRK cells became less well-spread and irregularly shaped whereas NIH 3T3 cells lost most of their stress fibers with an increase in locomotion rate when they were cultured on increasingly compliant substrates.

Cell movement can also be guided by the manipulation of flexible substrates to produce mechanical strains in polarized cells. When NIH 3T3 fibroblasts were cultured on flexible polyacrylamide sheets with type I collagen coating and transition in rigidity [29], cells approaching the transition region from the soft side could easily migrate across the boundary whereas cells migrating from the stiff side turned around or retracted as they reached the boundary. Cell also spread to a greater extent on stiff substrates compared with more compliant counterparts [30].

Contractile myocytes sense the mechanical as well as molecular microenvironment. Myoblast culture has been studied on collagen strips attached to glass or polymer gels of varying elasticity [31]. Myosin/actin striation emerges only on gels with stiffness typical of normal muscle. Adhesion strength increases monotonically with increasing substrate stiffness.

Effect of Chemical Properties

The chemical properties of a polymer have an important role in its surface functionality and consequently its cell behavior. When cells are exposed to a polymeric surface, a layer of protein adsorbs onto the surface within milliseconds. Thus, cells "see" the adsorbed protein layer rather than the actual polymer surface.

The surface chemistry of a polymer may be fine-tuned to control protein adsorption, which in turn controls cell adhesion. Depending on the desired outcome, the surface chemistry of a polymer can be modified to modulate the interactions of the adherent cells, such as morphology, migration, differentiation, proliferation, and apoptosis. In the context of biointeractions, important chemical properties of a polymeric surface may be categorized into its surface wettability and charge.

Surface Wettability

The wettability of a polymer surface is a measure of its hydrophobicity or hydrophilicity, or its surface energy. Water molecules at a polymeric surface rearrange around proteins, causing the native protein to unfold and adsorb irreversibly to the surface. Water molecules are unable to form hydrogen bonds with hydrophobic substrates. Hence, they form hydrogen bonds within themselves, leading to a more ordered structure with lower entropy. Proteins present in the serum can act as surfactants in which their hydrophobic domains interact with the substrate and their hydrophilic domains form hydrogen bonds with the water molecules. This results in release of the ordered water molecules, which is energetically favorable owing to the increase in entropy. This is known as the hydrophobic effect [32]. In general, proteins preferentially adsorb onto a hydrophobic surface, as mediated by their hydrophobic domains. The adsorbed protein monolayer is seen by the cells instead of the underlying surface, modulating adhesion to a great extent. For example, fibrinogen adsorbed onto a polymer surface greatly increases platelet adhesion.

Functionalization of polymer surfaces with poly(ethylene oxide) (PEO) creates a hydrophilic surface that easily becomes hydrated. PEO is the standard for creating a hydrated hydrophilic surface and is commonly employed to reduce uncontrolled protein adsorption or biofouling onto an implant device.

Surface Charge

The surface charge of a polymer affects protein adsorption and unfolding on its surface. Unlike surface wettability, the driving force for protein unfolding onto a charged surface is ionic interactions, not hydrophobic interactions. Protein unfolding depends on the net charge that proteins and cells encounter on the surface. For example, PEO is hydrophilic but has a net neutral charge. In contrast, NH_2 and COOH groups become ionized in solution, resulting in a net positive and negative charge, respectively. Many proteins have a net negative surface charge, which promotes their adsorption onto a positively charged surface. In addition, the glycocalyx on a cell surface (the polysaccharide mucosal layer) has a largely negative charge, adhering to positively charged surfaces via nonspecific interactions.

Cellular Response

The effect of surface chemistry on cellular behavior begins at the point of interaction. The surface chemistry influences the pattern of protein immobilization, absorptive or ionic, on the surface. For example, polymers with higher hydrophobicity are demonstrated to promote greater osteogenesis (bone regeneration) in vivo [33]. This effect has been attributed to a more favorable balance between hydrophobic and hydrophilic properties, which promotes greater protein adsorption onto its surfaces as well as enhanced cell adhesion.

Hydrophilic surfaces appear to inhibit leukocyte adhesion and the attached cells exhibit a decreased cytokine response. This results in an attenuated inflammatory reaction and decreased macrophage fusion [34]. Thus, hydrophilic polymer surfaces may offer an approach to limiting leukocyte adhesion and consequently improving the biocompatibility of an implant.

Surface chemistries also regulate the conformational changes in binding domains of FN, directing integrin binding affinity and the specificity of cell adhesion. In turn, this provides a greater degree of control over cellular behavior. For example, $\alpha_5\beta_1$ integrin was shown to bind with greatest affinity to hydrophilic, noncharged surfaces (OH functionalized end groups), intermediate affinity to hydrophilic, charged surfaces (NH_2 and COOH end groups), and least affinity to hydrophobic surfaces (CH_3 end groups). In contrast, $\alpha_V\beta_3$ integrins bind with highest affinity to COOH surfaces, intermediate affinity to NH_2, and negligible affinity to OH and CH_3 modified surfaces. These differences in binding and adhesion were reflected in the varying degrees of mineral matrix deposition and osteoblast differentiation of MC3T3-E1 cells [35].

Methods of Altering Surface Chemistry

Modifications of the surface chemistry of a biomaterial allow for the selective treatment of the superficial layer without changing its bulk property. This is achieved mainly through coating or by plasma treatment. A note of caution on oxygen plasma treatment of a polymeric substrate is that it might have an unintended effect on the physicochemical properties of the polymer. For instance, polydimethylsiloxane (PDMS) is a popular substrate for many studies of cell–substrate interactions because of the ease of fabrication and the optical transparency of the polymer. The stiffness of the substrate can also be fine-tuned by varying the cross-linking density. However, oxygen plasma treatment of PDMS can affect its surface chemistry and consequently the topography and elasticity at the nanoscale level. At low cross-linking density, it is also important to extract the unreacted cross-linkers before initiating the cellular studies [36].

Coating or deposition of a top layer includes several methods. Solvent coating or casting is a method in which a polymer is dissolved in a solvent (usually an organic solvent), which in turn is then soaked, brushed, or sprayed onto a surface. Polyelectrolyte multilayers [37] are generated by depositing alternating layers of polycationic and polyanionic monolayers onto a surface. Self-assembled monolayers (SAMs) consist of chemisorbed monolayers of closely packed alkanethiols onto surfaces such as gold, silver, or mercury. The head groups with a hydrocarbon tail may be functionalized with different end groups such as hydrophobic CH_3, hydrophilic OH, or charged COO end groups [38]. Polydopamine coating has proved effective in modifying the surface functionality of a biomaterial [39]. This versatile method applies to any base materials as well as to various conjugation chemistries. For example, Yang et al. applied polydopamine coating on 316L stainless-steel stents that demonstrated a differential response of the coating for human umbilical vein endothelial cells (HUVECs) and human umbilical artery smooth muscle cells (HUASMCs). Polydopamine-coated stents increased HUVEC adhesion, proliferation, and migration while suppressing the adhesion and proliferation of HUASMCs, thus leading to faster reendothelialization [40]. In another study, 3D tissue engineering of nanofibrous scaffolds of PCL were modified to coat the fibers with polydopamine [41]. These scaffolds allowed HUVEC cells to attach well and spread with a high cell viability. Chuah et al. conjugated the polydopamine on PDMS surface to fabricate a substrate for long-term culture of mesenchymal stem cell (MSC) adhesion and proliferation [42]. This effect was attributed to the change in the surface wettability of PDMS and the presence of hydroxyl and secondary amines on the surface compared with uncoated surfaces. Polydopamine-coated substrates have also proven to be highly efficient in supporting the reprogramming and self-renewal of human pluripotent stem cells (hPSCs) under defined conditions [43].

Another method of altering polymer chemistry is via plasma treatment of the surfaces of interest. Plasma treatment creates ionized gases such as ions, free radicals, and electrons from electron and ion impact in an electric field. These ionized particles create oxidized and groups on the polymer surface by breaking chemical

bonds. In surface etching, inert gases such as argon are employed to remove impurities and increase surface roughness. In addition, plasma etching allows the alteration of surface reactivity by cross-linking polymer chains.

Effect of Biological Properties

Although the physical and chemical properties of the biomaterial have an integral role in modulating cell behavior, biological features may be equally important if not so, because they represent the natural ECM. Thus, bioactive molecules such as adhesion ligands, growth factors, and enzymes may be physically entrapped, surface-immobilized, or covalently conjugated onto the substrate to resemble the ECM and control cell behavior.

Commonly Used Ligands

Bioactive substrates can be produced by the surface immobilization of a large variety of ECM cell adhesion proteins such as FN, vitronectin, laminin, and collagen. With the advent of molecular biology tools, the amino acid sequences of the specific cell-binding sites of these proteins have been identified, which has led to the development of short peptide ligands for the modulation of critical cellular processes. The Arg-Gly-Asp (RGD) tripeptide is a commonly occurring motif present in several cell adhesion glycoproteins; it mediates binding to specific members of the integrin family (FN and vitronectin bind via RGD to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins, respectively) [44]. The YGISR peptide derived from laminin binds to a family of nonintegrin cell adhesion receptors and can induce cell adhesion and motility [45]. Besides the RGD motif, substrates coated with short cell adhesion peptides are increasingly being employed to develop biomimetic substrates. Short peptides offer several advantages over entire ECM proteins. They are chemically defined and can be used in higher densities. They possess diverse functions because they can provide cell adhesion sites and matrix binding sites, promote self-assembly and growth factor binding, or a combination of all of these. Several new peptide ligands have been identified by using peptide libraries and de novo design. Overall, application of peptides instead of entire proteins eliminates immunogenic risks, facilitates conjugation without the loss of bioactivity, and improves cell adhesion and selectivity to the scaffold.

Cell surface proteoglycans have also been known to bind to proteins containing a large number of cationic amino acids. This finding has led to the generation of substrates modified with a positively charged surface.

Development of Bioactive Surfaces

Based on the naturally occurring hydrophobic effect of proteins in solution, several strategies have been developed to immobilize either naturally occurring ECM proteins or ECM-derived peptides (cell adhesion ligands) by adsorbing them directly onto a substrate. Covalent conjugates of nonadhesive bovine serum albumin (BSA) and RGD peptide adsorbed via BSA onto the surface of tissue culture plates have supported cell adhesion [46]. The disadvantage of adsorption-based methods includes easy desorption of the bioactive molecules by other proteins such as antibodies that may be routinely employed in assay procedures. More important, protein adsorption may cause the burial of the active site of the adhesion ligand, rendering it inaccessible for cell binding. Furthermore, protein adsorption may constrain the peptide in a conformation that reduces its binding affinity [47].

Challenges of bioactive surface development have been overcome by new methods of protein/peptide immobilization in which peptides are covalently grafted onto surfaces that typically do not support cell adhesion. These techniques allow the precise control and quantification of ligand density. Furthermore, the inert nature of these substrates ensures that any cell adhesion observed can be attributed solely to the covalently bound peptide. Several methods have emerged for covalent grafting of proteins or peptides onto the surfaces. Carbodiimide chemistry is a widely used method to conjugate molecules covalently containing amine groups (proteins and peptides) with carboxylic acid–containing substrates [48]. However, carbodiimide chemistry is nonspecific and cannot target a particular reactive group on the biomolecule structure and the substrate. Therefore, the binding of peptide or protein chains to the substrate can occur at multiple locations, which can prevent them from assuming the correct configuration and affect the activity of the protein or peptide.

Click chemistry-based covalent grafting is being increasingly used, because the reactions are highly efficient, region-selective, and orthogonal in nature [49]. Photoinitiated reactions can also be used to bind ligands to the substrate. This technique involves functionalizing the ligand of interest with a heterobifunctional cross-linker containing a photoreactive group, which can be subsequently conjugated with the substrate upon exposure to light [50].

Probably the most significant advantage of using phototriggered reactions is the ease with which spatial organization of the ligands can be achieved in 2D and 3D substrates.

Bioactive surfaces with immobilized ligands of varying concentrations modulate cell response differently. For instance, human foreskin fibroblasts seeded on glycophase glass-modified substrates exhibit increasingly spread-out morphologies with higher ligand concentrations. Higher ligand concentrations also generate more focal contacts and stress fibers. These findings thus established that RGD spacing of 440 and 140 nm is sufficient to promote cell spreading and cell adhesion, respectively [51]. Glycophase glass-modified substrates specific for laminin demonstrated both cell attachment and spreading of different cell types via nonintegrin cell adhesion receptors. In contrast, surfaces containing adsorbed laminin supported only cell attachment [47].

Another approach to generating nonadhesive substrates derivatized with cell-specific ligands is to employ the nonadhesive polymer poly(ethylene glycol) (PEG). Graft copolymers of RGD-modified PEG and poly-L-lysine could undergo surface immobilization onto negatively charged surfaces via electrostatic interactions. These surfaces could then support the binding and spreading of human dermal fibroblasts [52].

Alkanethiolates bind to gold and silver monolayer surfaces and form SAMs. Alkanethiolates modified terminally with the biologically inactive oligo(ethylene glycol) (OEG) have been extensively used in studying the modulation of cell behavior by substrates based on mixed SAMs. Mixed SAMs consisting of alkanethiolates modified terminally with either RGD or OEG promoted cell attachment via the cell adhesive RGD or cell repulsion by the OEG regions [53].

Finally, chemisorption is an immobilization strategy inspired by the composition of adhesive proteins in mussels [39]. The coexistence of catechol (L-dopa) and amines (lysine) in mussel pads was crucial for adhesion. Therefore, dopamine, a naturally occurring catecholamine containing both catechol and amine functional groups, was used as a bioadhesive building block for multifunctional surface coatings. To immobilize proteins or peptides, a polymerized dopamine layer is first formed on the substrate, which can then immobilize the molecule of choice.

Cell–Bioactive Surface Interactions

The property of the underlying ECM and its corresponding ligands determine cell behavior and response. Thus, the desired application (to evoke cell adhesion and proliferation versus cell migration and differentiation) may be tailored by choosing and designing the appropriate ligand, which will enable an engineered cell response. This, in turn, can affect the substrate itself, similar to the cell–ECM adaptive behavior inherent in vivo. The effects of some ligands on various aspects of cell behavior are presented next.

Cell Adhesion

Cell adhesion and its natural outcome of cell spreading are the first interactions that occur between the cell and the ECM. Cells adhere to the underlying ECM via cell–substratum bonds, which are typically receptor–ligand complexes formed between adhesion receptors (such as integrins) and their ligands (such as FN). Features of the substratum such as ligand density have been shown to affect cell adhesion and spreading via intracellular mechanisms that have not been completely delineated.

The RGD motif in the ECM proteins is an important ligand for cell adhesion. The ability of the RGD peptide to affect cellular adhesion depends on its activity, which can be modulated by the presentation of cell-binding complementary domains. Because ECM molecules exhibit multiple integrin interaction domains, spacing between these domains affects cell adhesion and migration events. For example, cells cultured on recombinant FN fragment encompassing the seventh through 10th type III repeats that presented the Pro-His-Ser-Arg-Asn (PHSRN) and RGD sequences ($FNIII_{7\text{-}10}$) in the correct structural orientation exhibited significantly higher adhesion strength and FAK activation versus surfaces modified with RGD ligand alone [54]. Interestingly, in the same study, separating the RGD and PHSRN ligands by a polyglycine linker did not improve the cellular adhesion strength compared with the $FNIII_{7\text{-}10}$ fragment, which underscores the importance of properly spacing ligands.

The immobilization strategy can also affect the integrin–ligand interaction. FN immobilization on an ammonia plasma-treated poly(tetrafluoroethylene) (PTFE) surface using glutaric anhydride promoted optimal display of the RGD motif, as opposed to a sulfosuccinimidyl-4-(*p*-maleimidophenyl)butyrate linker [55]. However, RGD peptide may not be the best choice of ligand in every case to promote cellular adhesion. In another study, the RGD, YIGSR, and IKVAV domains of the ECM protein laminin were grafted onto an aminated PCL surface [56]. The results revealed that the IKVAV sequence provided the highest cellular adhesion for human adipose–derived stem cells. Gauvreau and Laroche used their aminated PTFE surface to print different combinations of the cyclic RGD, cyclic Gly-Arg-Gly-Asp-Ser (CGRGDS), and cyclic H-Trp-Gln-Pro-Pro-Arg-Ala-Arg-Ile (CWQPPRARI) peptides,

all derived from FN [57]. They found that a combination of GRGDS and WQPPRARI were most efficient in promoting the attachment, spreading, and proliferation of HUVECs.

The length of the ligand spacer arm can also influence ligand–receptor interaction. In one study, alginate gels were modified with RGD peptides of varying spacer arm lengths. The spreading of the primary fibroblasts was directly proportional to the number of glycine units in the spacer arm of the RGD peptides, up to 12 glycine units. Increasing the spacer length from 12 to 20 glycine units did not increase the cell spreading further [58]. Similarly, the type of the spacer used affects the binding affinity of the integrin receptor to the RGD ligand–polyproline spacer (compared with an aminoethoxyacetic acid or PEG-containing spacer), resulting in ligands with higher integrin-binding affinity and improved cell adhesion properties [59].

A large number of studies have been conducted to delineate the relationships among ligand density, spatial arrangement, and their effect on cellular adhesion. Previously, the effect of ligand density on cell adhesion was studied by varying the concentration of ligand solutions in which ligands were present ubiquitously on the substrates. Hepatocytes cultured on surfaces expressing interstitial ligands (laminin or collagen type I) or basement membrane (FN or collagen type IV) demonstrated an increased rate and degree of spreading at higher ligand densities compared with lower coating densities. This direct dependence on ligand density was attributed to the increased number and density of cell adhesion receptor–ligand bonds, which may generate forces that overcome cellular traction [60]. Alternately, the formation of focal contacts could be enhanced, leading to reorganization of the cytoskeleton. Initial cell adhesion was found to occur with an increase in actin microfilament (MF) mass and a concurrent, rapid cell-spreading phase. This was later followed by a slow rate of ECM-independent spreading. It was hypothesized that the MFs may have formed focal contacts with the ECM, which may provide the force for cell spreading. The fact that only a combination of cytoskeleton-disrupting drugs (targeting actin and microtubules) could inhibit cell spreading implies cytoskeletal redundancy, which means that one structural component can compensate for the other. Furthermore, the intracellular forces generated by focal contact formation may also be transmitted to the ECM [61].

Advanced fabrication techniques have enabled researchers to gain precise control over the arrangement of ligands in microsized and nanosized islands [62]. Contact printing can be used to prepare microsized islands of ligands of approximately equal size as FAs. On the other hand, receptor-sized ligand patterns can also be designed on substrates using techniques such as self-assembled nanoparticles, block copolymer micelle lithography, and electron beam lithography (EBL) [63]. By using such techniques, it has been shown that the formation of strong adhesive protein clusters requires extracellular adhesive ligands to be spaced closer than the critical value of around 60 nm [64].

Immobilized ligand gradients on substrates allow for the testing of a large range of ligand concentrations on a single substrate. A surface-immobilized osteogenic growth peptide (OGP) concentration gradient prepared by click chemistry was used to investigate osteoblast adhesion and proliferation. The highest cell adhesion occurred on areas of low OGP concentration (40–60 pmol/cm^2), whereas cell proliferation was higher on OGP gradients at all concentrations compared with the free ligand control [65].

Cell Motility

The migration of cells is an important aspect of cell behavior, especially during embryogenesis, organ regeneration, and other repair processes such as wound healing and angiogenesis. Main requirements for initiating cell migration are the breaking of existing cell–ECM bonds and the formation of new ones in the desired direction and at the next site of attachment on the substratum.

Studies on factors affecting cell motility such as ligand concentration have shown that the migration speed of human MSCs (hMSCs) on FN or collagen IV-coated surfaces has a biphasic dependence on ligand concentration. The maximal speed is attained at intermediate ligand density. This suggests that very low densities do not provide the traction needed for movement whereas extremely high densities confer strong cell adhesiveness that deters cell detachment from the substrate. Because the shear forces for cell detachment for FN and collagen are similar, it was also concluded that the strength or adhesiveness of the initial cell–substratum bond governed cell migration speeds [66]. Studies have also shown that the high ligand density reduces the cell migration rate on 2D surfaces. In contrast, cell migration on a 1D surface was found to be independent of ligand density [67]. The study concluded that contact guidance provided by the linear topographical cue compensates for the prohibitive effect of high ligand density on cell migration. Studies have shown that contact guidance can improve the cell migration rates. Interestingly, the cell migration in the 3D fibrillar matrix was similar to the 1D matrix and both depended on the myosin II contractility.

The concentration gradient of ligands, particularly growth factors, has been studied to stimulate directed cell migration. Immobilized ligand gradients have been shown to stimulate directed keratinocyte migration [68]. In another study, a low-to-high concentration gradient of solid-phase heparin binding epidermal growth factor stimulated the migration of MSCs away from the cell source, which could potentially help in faster wound healing [69].

The initial bonds formed between the cell and ECM can also be affected by the affinity of the receptor to the ligand as well as the expression levels of the receptor itself. In one study, cell migration speed inversely depended on the expression and affinity of the integrin receptor. Maximal cell speed was achieved with intermediate ligand densities, integrin levels, and affinity. All of these parameters represent an optimal initial adhesiveness or an adequate number of cell–substratum bonds to support initial attachment followed by migration. Under conditions in which very few or too many cell–substratum bonds were formed, cells were observed to form short unstable lamellipodia or were too spread out to support movement. Interestingly, the maximal speed attained was found to be independent of ligand concentration, receptor expression levels, or affinity [70]. This suggests the involvement of other factors such as intracellular contractile force or the induction of intracellular signaling.

Because integrins have been shown to cluster and mediate downstream cytoplasmic signaling, the spatial arrangement of the ligand also influences motility. Murine fibroblasts were grown on surfaces that presented the YGRGD ligand either singly or in clusters while maintaining the same average ligand density for the entire surface. Cells showed higher adhesion strength and migration speeds when the ligand was presented in increasing cluster numbers compared with single ligands. Furthermore, the maximum speed attained by the clustered ligands was achieved with lower overall ligand densities compared with the higher ligand densities required when ligands were expressed singly. Finally, the presence of stress fibers and distinct FAs in cells exposed to the clustered ligand confirmed the importance of the orientation of the ligands in cell adhesion and motility [71]. Well-designed surfaces with precise control of ligand presentation and interligand distance allow for a mechanistic understanding of ligand–receptor interaction and its effect on cell migration. With the invention of nanotechnology tools, significant progress has been made in the design and fabrication of surfaces with precise and controlled ligand presentation. In a study, gold RGD-immobilized nanodots were prepared with 58 or 108 nm interligand spacing [72]. Fibroblasts formed stable FAs on the 58-nm spacing surface, which resulted in stationary cells. On the 108-nm spacing surface, however, the cells had higher migration rates, which were associated with the high turnover of FAs. Furthermore, the molecular properties of FAs were dissimilar on differently spaced ligands: e.g., the presence of zyxin, an FA protein, was significantly lower in FAs formed on the 108-nm spacing surface [72]. Another study showed that cells can sense ligand spacing variations as low as 1 nm and use this information to induce cell polarization and cell migration [73].

Integrin-mediated cell migration is the universal mechanism employed by cells with the exception of leukocytes. Leukocyte migration in 3D environments occurs independently of integrin-mediated migration. Instead, these cells migrate by the sole force of actin-network expansion, which promotes protrusive flowing of the leading edge. Integrin-mediated adhesion and migration are necessary only when cells need to overcome tissue barriers such as the endothelial barrier [74].

Cell Proliferation, Self-renewal, and Differentiation

Cell–ECM interactions can also modulate cell function, although the underlying mechanism remains unclear. Identification of critical parameters such as cell–cell contacts, key proteins, and intracellular tension will allow their incorporation into an artificial ECM that can stimulate desired cell function when necessary.

Because stem cell phenotype and fate are influenced by the interaction of cells with the niche, many studies have focused on investigating the effect of ECM ligand type, density, and spatial arrangement, which provide stimuli for directing the differentiation of stem cells into specific lineages. Similarly, self-renewal of stem cells is regulated by several ECM molecules. A study showed that although FN has a vital role in the proliferation and self-renewal of mouse embryonic stem cells (mES), excessive FN levels could result in cell spreading and differentiation [75]. Laminin-511 is another important ECM ligand that supports mES attachment, proliferation, and pluripotency [76]. Interestingly, this study showed that other forms of laminin, such as -332, -111, or -411, were not effective in maintaining cell adhesion and self-renewal [76]. Subsequent studies showed that recombinant laminin-511 supports attachment and proliferation and maintains the pluripotency of human embryonic stem cells (hESCs) as well as induced pluripotent stem cells (iPSCs) by binding to $\alpha_6\beta_1$, an integrin that is frequently expressed by these cells [77]. Other integrins that can maintain the pluripotency of iPSCs include $\alpha_V\beta_5$ integrin via vitronectin binding [78].

Stem cell differentiation is also affected by the type of ligand, density, and presentation. Studies have shown that a combination of ligands could modulate the differentiation of stem cells, which was not achieved with the use of a

single ligand molecule. For example, cell culture substrates functionalized with peptide ligands for $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrin efficiently supported the early mesodermal differentiation of hESCs. Cellular differentiation, however, could not be induced in the same cell culture medium with the use of either ligand alone [79]. The spatial presentation of ligands is another factor that could regulate cell differentiation. Increased lateral spacing of adhesion peptide induced the increased adipogenic differentiation of MSCs whereas the smaller spacing of ligands resulted in the osteogenesis of MSCs [80]. These results established the strong effect of nanometer-scale changes in ligand arrangement on stem cell differentiation.

Cell shape has been long proved to be a vital regulator of cell function. Bioactive surfaces can be tailored to modulate cell shape by controlling the ligand density or arrangement (to create patterns) on the surface. Hepatocytes cultured at very low initial densities (to minimize cell–cell interactions) on substrates coated with different densities of FN, collagen type I, laminin, or collagen type IV would exhibit a density-dependent alteration of cell shape. The lowest densities were sufficient to support cell attachment but not promote cell spreading, as evident from the rounded cell morphology. In contrast, higher densities mediated a shift to extensive cell spreading. These changes in cell shape were accompanied by corresponding changes in cell function. Rounded cells observed on low-density surfaces exhibited a lower degree of proliferation and bore signs of differentiation, such as the increased production of liver-specific proteins such as albumin. On the other hand, highly spread-out cells on high-density surfaces tended toward proliferation rather than differentiation [81]. The density of ligands influences the number of cell–ECM bonds formed and consequently cell shape. This, in turn, may regulate the switch between proliferation and differentiation via several possible mechanisms such as the clustering of the integrin receptors leading to cytoskeletal reorganization, upregulation of downstream signaling pathways, or the distribution of intracellular forces.

The effect of cell shape on function was also convincingly demonstrated when hepatocytes were grown on laminin-coated adhesive islands (2–80 μm) surrounded by nonadhesive PEG areas. Primary rat hepatocytes were restricted to these adhesive islands and adopted the underlying square or rectangular island morphology, as opposed to cells showing pleiomorphic forms when grown on nonpatterned adhesive surfaces. This confinement of cell shape led to increased cell differentiation (albumin secretion) with a concomitant reduction in growth. Because the ligand density was maintained over the various islands, this regulation of cell function could not be attributed to a lack of cell–ECM contacts but primarily to cell shape, and consequently triggered molecular pathways [22].

Effect of Topography

Cells in tissues or organs respond to organized spatiotemporal stimuli. During the development of an embryo, the surrounding ECM provides a hierarchical organization of topography that ranges from meso to molecular scales. Topography, coupled with biochemical and physical cues, regulates cellular functions such as migration, adhesion, morphogenesis, differentiation, and apoptosis in a developing embryo [82]. Defined topographical cues allow the systematic study of cell–substrate interactions (termed contact guidance) and can also modulate cellular orientation and morphology that may in turn be extended to modify other cellular responses.

Fabrication Techniques

To study the effect of topography on cellular behavior, patterning techniques have been developed to create defined substrate topography at the micron scale. With further advancements in patterning techniques, topographical structures may be fabricated at the nanometer scale over larger areas and with greater ease [83]. Fabrication at nanometer dimensions is highly relevant because most in vivo structures are found at the nanometer scale, such as the 40- to 120-nm collagen fibrils of the basement membrane.

Several reviews have been published on common techniques used to generate topographically modified surfaces to study cellular behavior [84,84a]. Many techniques have been developed from photolithography. One of the first studies using photolithography to fabricate patterned structures involves coating a resist onto a surface and subsequently exposing it to UV light through a patterned mask [85]. Thus, a photochemical reaction occurs only at the exposed areas of the resist. These reacted areas of the resist are either retained or dissolved away when soaked in a developer solvent, depending on whether a positive or negative resist is used. Photolithography allows the facile fabrication of submicron-sized topography with great reproducibility over large areas. However, the size of the feature achieved is curtailed by the wavelength of light diffraction. This limitation may be overcome by EBL to fabricate fine features at the submicron level. However, this method is both expensive and time-intensive.

TABLE 27.1 Commonly Used Two-Dimensional Substrate Patterning Techniques for Tissue Engineering and Regenerative Medicine Applications

Technique	Materials That Can Be Patterned	Advantages	Disadvantages
Nanoimprinting	Thermoplastic polymers, polymer resists	One step patterning of thermoplastics Very high resolution (<50 nm) High throughput Cost-effective	Requires a master mold Requires etching step to transfer patterns for substrates other than thermoplastics
Photolithography	UV-sensitive prepolymers	Does not require a master mold Well-suited for low-resolution, UV–cross-linked hydrogel patterning	Low-resolution (micron range) Limited to hydrogel patterning unless combined with etching step or soft lithography
Hot-embossing	Thermoplastic polymers	One-step patterning of thermoplastics Cost effective	Requires a master mold
Injection molding	Thermoplastic polymers	Rapid and low-cost operation High throughput	Higher capital cost Limited to micron resolution Requires electroformed master dies [89]
Electron beam lithography (EBL)	Spin coated EBL resists such as poly(methyl methacrylate)	Excellent resolution (<10 nm) Master mold is not required High-nanoscale reproducibility	High capital cost Slow patterning Requires etching and embossing step to transfer patterns to cell culture substrate
Soft lithography (molding)	Polydimethylsiloxane, hydrogels, protein solutions	Simple process, cost-effective Applicable to wide range of materials	Requires a master mold Challenging to obtain nanoscale resolution

Patterns obtained by photolithography may be transferred to elastomeric molds such as PDMS. This technique, known as soft lithography, allows structures as small as 30–50 nm to be fabricated [83]. Soft lithography can also be used to "stamp" patterns onto a surface; this is known as microcontact printing [86].

Polymer demixing, which is based on phase separation, can also generate nanoscale structures. For instance, polystyrene and poly(4-bromostyrene) were shown to demix spontaneously, producing islands that varied from 13 to 95 nm in height, depending on the ratio of polymer to solvent mixture [87]. Although this technique is simple and inexpensive, its main disadvantage is a compromise in precision.

Electrospinning [88] has emerged as a simple, efficient method to produce polymer fibers as scaffolds for cell and tissue engineering. Briefly, electrospinning uses a high-voltage field to overcome the surface tension of a polymer solution, to form fibers that are deposited onto a grounded surface. Electrospinning yields a nonwoven mesh that, if collected over a longer time, can form a nonwoven 3D scaffold. Alignment of the fibers can be accomplished by spinning the grounded surface at the same speed as the rate of fiber deposition (Table 27.1).

Cellular Responses to Topographical Cues

The complex cross-talk among cell adhesion molecules and the ECM, intracellular and ECM-generated mechanical forces, and biochemical signaling molecules, elucidates a correlation between cell shape and function [90]. Control of cell morphology dictates cell behavior such as growth, differentiation, and survival. Topographical cues can induce changes in cell morphology, affecting cellular responses such as proliferation, gene expression, cytokine production, and cellular function. These responses vary depending on the cell type and the geometry and size of the topographical features; they are reviewed elsewhere [91,92]. It has been suggested that all cell types will attain a spherical morphology with a less-organized cytoskeleton when exposed to isotropic features 5 μm or smaller in dimension [93].

Disordered topographies on poly(methyl methacrylate) (PMMA) substrates have an important role in cell differentiation and aggregation. Varying the degree of randomness in nanopit spacing was shown to affect cell adhesion and increase the expression of bone-specific ECM [94]. Interestingly, a range of semirandomness of the nanopits was shown to provide optimal enhancement in cell adhesion and promote bone-specific ECM.

The importance of the feature size was illustrated when smooth muscle cells were cultured on PDMS and PMMA surfaces presenting a range of different-sized grooves. Cells exhibited superior alignment along the grooves of surfaces with the smallest topographical features. Epithelial cells aligned on uniform grooves and ridges showed greater adhesion strength when the groove size was reduced from 4000 to 400 nm by exposure to fluid shear stress [95]. Illustrating the effect of feature size on cellular behavior, it was shown that cells grown on adhesive, patterned islands of varying sizes and shapes conformed to the underlying substrate geometry.

At the nanometer scale, uroepithelial cells cultured on hemispherical pillars or step edges demonstrated a stellate morphology compared with the spread-out morphology adopted when grown on smooth surfaces. The less-spread morphology also correlated with a decrease in cytokine production. Interestingly, uroepithelial cells cultured on parallel grooves and ridges showed only differences in morphology while cytokine production remained unaffected [96]. Furthermore, smooth muscle cells showed a decreased rate of proliferation when cultured on nanoimprinted gratings compared with those cultured on nonpatterned surfaces [97], which demonstrated the effect of nanotopography on cell morphology, proliferation, and migration. Muhammad et al. also showed that nanograting topography on titanium oxide substrate suppressed the proliferation of human coronary artery smooth muscle cells [98]. In another study, gratings with line widths of 350 nm and 1 and 10 μm on PDMS were used to study the differentiation of hMSCs into neuronal lineage, with or without retinoic acid induction. The expression of microtubule-associated protein 2 (MAP2) was most significantly upregulated on 350-nm gratings compared with the 1- and 10-μm gratings. A synergistic effect of nanotopography and retinoic acid was also observed on the upregulation of neuronal marker, compared with the induction of retinoic acid or of topographical alone [99]. These studies further illustrate the significance of topographical feature size on cellular responses.

Neuronal cells present a unique response to topography. Unlike other cell types, neurons have certain distinctive cell shape features. A typical neuron is a polarized cell with soma and dendrites on one end and axons on the other. Neurite alignment or outgrowth was shown to increase with increasing groove depth from 0.2 to 4 μm. With isotropic pillars, hippocampal neurons could expand from pillar to pillar in a highly ordered manner when interpillar spacing was less than 40 μm [100].

Multipotent neural stem cells (NSCs) are highly interesting to regenerative medicine and neuroscience studies because they are present in both developing and adult central nervous system. The NSCs are capable of self-renewal and differentiation into astrocytes, oligodendrocyte, and neurons whereas the neural progenitors (NPCs) are lineage restricted and capable of fewer divisions. The major types of progenitors found in the adult human brain are ventricular zone NPCs, hippocampal neuronal progenitor cells, and white matter glial progenitors [101–108]. Many studies have expressed the significant influence of topography on NSC and NPC differentiation, proliferation, morphology, and even regional specification. Christopherson et al. demonstrated the application of electrospun polyethersulfone fiber mesh coated with poly-L-ornithine and laminin for the maintenance of rat hippocampus-derived adult NSC [109]. That study showed that as the diameter of the fiber mesh decreased from 1452 to 283 nm, the proliferation and spreading of the NSCs increased and exhibited lower aggregation. Furthermore, the fibers were more efficient at promoting differentiation when the NSCs were cultured under differentiation conditions. Moe et al. studied the influence of different geometries and sizes in neural differentiation of primary murine NPCs (mNPCs) using a microarray of various topographical structures as a Multi-ARChitecture chip [110]. The customizable microarray contains distinct topographies of various architectural complexities, from nanometer to micrometer dimensions, with different aspect ratios and hierarchical structures on a single chip. They showed that anisotropic gratings significantly promoted differentiation of the stem cells into neurons. In contrast, glial differentiation was enhanced on isotropic holes and pillars. Interestingly, a similar trend of neuronal differentiation of hESCs was shown by Ankam et al. [111]. In their study, the anisotropic nano- and microgratings enhanced neuronal differentiation and maturation of hESCs. These studies showed that the geometry and size of a topographical structure can influence the lineage commitment of the stem cells, even when the cells were cultured under the same condition. Tan and Tann et al. took one step further to investigate whether topography affects regional specification in the derivation of midbrain dopaminergic neurons [112]. The gratings and hierarchical gratings, which were composed of a secondary set of nanogratings imprinted on top of microgratings, showed the most significant enhancement in the midbrain dopaminergic neuron derivation.

The aspect ratio of the height and width of topographical features is also an important parameter in guiding neurites. Different depths of grooved substrates have been studied systematically and have been shown to have an effect on cell morphology [113] and neuronal differentiation [114]. Neurons differentiated from mNPC on groove substrates of different depths (0.35, 0.8, 2, and 4 μm) revealed that an aspect ratio of height to width > 1 would enhance neurite growth. With a height to depth aspect radio < 1, neurite alignment parallel to the grooves decreased. A model was proposed to hypothesize that the depth-sensing ability of the neurites is involved with the interplay

between filopodia adhesion and neurite bending. Thus, the size of topographical features, ranging from nanoscale to microscale, has been shown to have a role in directing neural differentiation and neurite guidance.

In addition to stem cell differentiation, neurite guidance, and morphological changes, topographies affect and regulate other cellular responses. Cells stretched by the underlying substrate were observed to switch from an apoptotic mode to growth [115]. The geometry of the underlying substrate was also found to affect fibroblast attachment, in which the greatest adhesion occurred at ridges but diminished on nanosized pits and columns [116]. Differences in proliferation and adhesion can lead to differential endocytotic activities of cells on topographies. Human fibroblasts attempted to endocytose nanocolumns 100 nm in diameter fabricated using colloidal lithography [117]. Another work demonstrated that the topographical effects influence endocytosis, and in turn the efficiency of drug and gene delivery in hMSCs and fibroblasts [118]. The enhanced cellular endocytosis exhibited by the cells on micro- and nanopillars promoted the internalization of fluorescently labeled dextran, as well as the nonviral gene transfection of green-fluorescent protein encoding plasmid with lipofectamine. By optimization and careful design, various topographies can be applied to regulate drug and gene delivery and cell proliferation.

Corneal endothelial cells generally have low in vitro proliferation rates and are nonproliferative in vivo. However, studies demonstrate that appropriate surface topographical cues can induce higher proliferation rates in corneal endothelial cells [119] as well as in primary human corneal endothelial cells [120]. Furthermore, topographical cues increased the expression of the tight junction protein zona occludens 1 (ZO1) and promoted in vivo cell-like morphology [120]. The study also demonstrated that the donor-derived human corneal endothelial cells were able to memorize the topographical cues such that even after passaging, the cells could consistently express increased ZO1 and improve cell morphology.

Pathological changes to the microstructure of basement membranes can occur under pathological conditions. Fuchs endothelial dystrophy (FED) is one such disease that is characterized by the development of microdomes and pillars-like structures known as corneal guttata on the corneal endothelial basement membrane. Rizwan et al. characterized the guttata dimensions at various stages of FED disease and then developed synthetic guttata substrates as an in vitro disease model. This model was subsequently used to study the monolayer formation and migration of primary corneal endothelial cells on guttata-like microtopography [121]. This study is an example of how in vitro disease models can be used to mimic basement membrane microstructural changes and consequently to study the mechanisms underlying cell behavior in pathological settings.

Whereas many studies have demonstrated the significant influence of substrate topography on cellular responses, researchers have also been actively investigating the mechanisms underlying the topographical regulation of cell behavior. It has long been demonstrated that genetic expression can be influenced by surface topography. Human gingival fibroblasts aligned along 60-, 30-, and 15-μm grooves 5 and 10 μm deep on titanium-coated silicon demonstrated increased production of both cellular and plasma FN compared with nonaligned controls. The narrower grooves favored plasma FN whereas the wider grooves favored cellular FN [122].

How does topography affect gene expression? One working hypothesis is changes of integrin expression and/or clustering that subsequently affect FA signaling. The changes in hMSC integrin expression were studied using 350-nm patterned or unpatterned substrates [123]. A reduced expression of integrin subunits α_2, α_V, β_2, β_3, and β_4 was observed on nanopatterned substrates compared with unpatterned substrates. In addition, integrin expression was generally higher on TCPS than on PDMS substrates, which suggests that the substrate surface chemistry and stiffness were also contributing factors to integrin expression in hMSCs. Teo et al. showed that integrin-activated FAK is an early sensor and regulator of topography-induced hMSC differentiation [124]. FAK phosphorylation was required for topography-induced hMSC differentiation whereas FAK overexpression overruled the topographical cues in cell lineage bias. This work indicated a direct effect of FAK activity on topography-induced gene expression, explaining how hMSCs can sense and transduce topographical signals via FAK and cytoskeletal contractility to induce cell differentiation.

Actomyosin contractility could be another intracellular mechanism involved in topography-induced differentiation. Ankam et al. showed that high actomyosin contractility induced by a nanograting topography is crucial for neuronal maturation of hESCs during neuronal differentiation. Treatment of cells with the myosin II inhibitor (blebbistatin) and myosin light chain kinase inhibitor (ML-7) greatly reduces the expression level of MAP2 [125]. The mechanical signals are transduced through FAs via a mechanism involving vinculin, and into the contractile actomyosin cytoskeleton.

Another study identified zyxin as one of the molecular players involved in the topography-mediated modulation of cellular response [126]. When hMSCs were cultured on PDMS with 350-nm grating topography, downregulated zyxin expression led to smaller and more dynamic FAs. Because the association of zyxin with FAs depends on force, smaller zyxin-positive adhesions as well as their higher turnover rate suggest that the traction force in FAs on 350-nm topography is decreased. These changes led to faster and more directional migration on 350-nm gratings.

It also suggests that the 350-nm grating topography decreases intracellular tension. Interestingly, a higher turnover rate of zyxin was observed when cells were cultured on more compliant substrates [127]. Therefore, phenomenologically, the initial molecular response of a cell to nanotopography appears to mirror that of a compliant substrate.

Another crucial part of the mechanistic understanding of topography-induced differentiation is the effect on the stem cell differentiation efficiency and speed, and the crucial time period for applying topography to enhance stem cell differentiation. Chan et al. quantified the effect of topography on the neural differentiation of hPSCs during 21-day differentiation, and showed that gratings increased the rate of neural differentiation [128]. The group also compared the significance of a priming period in which the stem cells were cultured on the topography in maintenance media, with the actual contact of topography during the differentiation period. The hPSCs with both topography priming and topography contact during differentiation showed faster progress in differentiation compared with the culture with only the priming or topography contact during differentiation. The study demonstrated that topography contact is significant for topography-induced differentiation, and the effect of topography priming and topography contact is additive in enhancing differentiation.

In addition to stem cell–directed differentiation, a study showed that topography at the microscale can promote the direct conversion of fibroblasts into neurons [129]. When neuronal transcription factors Ascl1, Brn2, and Myt1l were overexpressed in murine fibroblasts, the fibroblasts could be transdifferentiated into functional neurons, the subtype of which depended on the topography of the PDMS substrate. The purity and conversion efficiency of the induced neurons (iN) were increased on micrograting substrates, whereas neurite branching was increased on microposts and decreased on micrograting. Compared with the reprogramming conducted on planar PDMS, the iNs reprogrammed on 5-μm micrograting showed upregulation of vasoactive intestinal polypeptide and downregulation of thymus cell antigen 1 and bone morphogenetic protein 5, and coupled with microarray DNA analysis, reflected a more gamma-aminobutyric acid-ergic rather than glutamatergic phenotype [129].

Although that study sheds light on the impact of topography at the cytoplasmic level, another study linked extracellular physical cues to changes in the nucleus [130]. When the same hMSCs were cultured on PDMS with 350-nm grating topography, 2D gel electrophoresis and western blotting showed that A-type lamins and retinoblastoma protein were downregulated compared with hMSCs cultured on planar PDMS. The differentiation potential of the hMSCs was also altered. When the hMSCs were cultured in mixed media containing adipogenic and osteogenic cues, the adipogenic markers peroxisome proliferator activator receptor γ and low-density lipoprotein in hMSCs cultured on 350-nm topography were downregulated whereas the osteogenic markers osteocalcin and Runt-related transcription factor 2 (Runx2) were upregulated compared with planar controls.

Electrically Conductive Substrate

Bioelectricity is the electrical currents and electrical potentials generated by or occurring within living cells, tissues, and organisms. For example, neurons conduct signals by using electrical fields. Similarly, cardiac tissue is composed of aligned cells and cardiomyocytes, which conduct the electrical current that provides signals for cardiac contraction and blood flow. The heart consists of Purkinje fibers, which are electrically conductive and essential for the conduction system of the heart. Because electrical current is an indispensable part of cell and tissue function, electrically conductive substrates and scaffolds for tissue engineering applications can be designed to facilitate better cellular cross-talk and tissue regeneration. Commonly used naturally occurring biopolymers or synthetic polymer scaffolds are not electrically conductive. These materials can be rendered electrically active by combination with conductive components such as carbon nanotubes (CNTs), carbon nanofibers, and gold nanowires. Shin et al. showed that the addition of CNTs into gelatin hydrogels improved cardiac cell adhesion and maturation, and promoted cell–cell electrical coupling [131]. The cardiac cells also showed synchronous beating on these CNT-incorporated hydrogels. These effects depended on the CNT concentration in the hydrogel, which indicated that the appropriate amount of electrical conductivity in a scaffold is an important parameter. Limitations of these electrically conductive scaffolds components are that they are nonbiodegradable and their long-term in vivo effects are largely unknown, which could present challenges for in vivo transplantation. This limitation may be addressed to an extent by blending conductive polymers such as polyaniline and polypyrrole with the scaffold material solution before processing. Polypyrrole has been shown to degrade in aqueous conditions [132] and polyaniline, although nonbiodegradable, can be used to synthesize biodegradable copolymers [133].

Besides endogenous electrical signals, externally applied electrical stimuli have long been known to modulate cellular functions and tissue healing. In the 1960s, Bassett et al. demonstrated that the mechanism of bone formation in adult dogs could be altered by applying direct electrical currents of varying intensities [134]. Since then, a

multitude of studies have confirmed the effect of external electrical fields on the cellular function of several tissues including heart, lungs, skin, and vascular and neuronal tissue. Schmidt et al. showed that neurite outgrowth could be enhanced by applying exogenous electrical fields on cells cultured on electrically conductive substrates [135]. In their study, cells exposed to either a constant potential or a constant current applied through polypyrrole film exhibited significant and uniform improvements in neurite extension with no directional bias. In another study, Rowlands and Cooper-White showed that proper exogenous electrical stimulation could enhance the proliferation rate of smooth muscle cells but also lead to increased contractile protein expression [136]. Conducting polymer substrates (polypyrrole) doped with hyaluronic acid and coated with collagen IV and Matrigel were used to mimic the basement membrane of smooth muscle cells.

A combination of electrical stimulation and a unique extracellular microenvironment can also be used to modulate cellular differentiation, regenerate tissues, and achieve faster wound healing. Cells can sense electrical stimuli as well as the characteristics of the physical microenvironment such as topography and stiffness. Therefore, electrically conductive scaffolds can be used in conjunction with physical cues to take advantage of the beneficial synergistic effects of physical and electrical cues and thus obtain greater control of cellular fate.

Carbon Nanotube and Graphene Surfaces

Carbon coatings have been established as a new biocompatible material that has no biochemically adverse effects on cells in culture [137]. Since this discovery, several studies on CNTs, graphene, and graphene oxide (GO) have been investigated, which have demonstrated a high potential for modulating cellular functions. Each of these materials provides an intrinsic set of physical, electronic, chemical, and mechanical properties and provides nanoscale dimensionality, cues, and texture [93].

The effects of CNTs are controversial because some studies have shown cytotoxic effects whereas others have shown increased cell adhesion and proliferation. It has been postulated that the line between adverse and augmented effects is based on the CNT length, degree of dispersion, and purity [138]. Multiwalled CNT (MWCNT)-soaked collagen was shown to improve initial cell attachment and proliferation of 3T3 mouse fibroblasts greatly. Increased adhesion was attributed to enhanced filopodia that wrapped around the CNTs. Because alkaline phosphatase activities were similar between cells that adhered to MWCNT-covered collagen and bare collagen controls, no significant effect on cell differentiation was expected [139].

Single-walled CNT (swCNT) coatings on surfaces have proved to increase cell adhesion and cell spreading of MSCs. MSCs preferentially adhered to swCNTs; after 1 week of culturing, they began to cross over to neighboring swCNTs. This favorable adhesion demonstrates nanostructure-directed growth that can be applied to neuronal culturing [140]. Furthermore, it has been demonstrated that mES can differentiate into neurons, astrocytes, and oligodendrocytes on a multilayered swCNT surface, which raises the possibility of a new potential surface for neural interfaces [141].

Graphene and GO surfaces have demonstrated accelerated osteogenic stem cell differentiation of hMSCs. The effect of a graphene coating does not appear to change cell adhesion or morphology on SiO_2 [142]; however, a comparison between bare PDMS and pure graphene and GO showed increased hMSC adhesion and proliferation on the carbon surfaces [143]. The accelerated osteogenic differentiation can be attributed to the special $\pi-\pi$ interactions, which allow for increased mineralization and an ability to preconcentrate osteogenic inducers such as dexamethasone and β-glycerol-phosphate [143]. GO has also demonstrated an increased capacity for absorbing serum proteins owing to its surface roughness and surface oxygen content [144].

A comparison of MWCNT, MWCNT/GO, single and multilayer GO, and reduced GO (rGO) on glass cover slips was performed using NIH 3T3 mouse fibroblast cells. The rGO and rGO/MWCNT had slightly increased cell adhesion after 48 h of incubation; however, the surfaces with MWCNTs had a larger cell area. The carbon-based materials exhibited an elevated amount of FAs, which implied a higher affinity for carbon versus the glass coverslip [145].

EFFECT OF DIMENSIONALITY

Research aimed at gaining a fundamental understanding of tissue development or designing a new biomaterial has always employed substrates that mimic the ECM as closely as possible. However, one aspect that has been more difficult to imitate is the 3D nature of the tissue. In the in vivo microenvironment, cells are not only surrounded by other cells, they are encompassed by the ECM (between the cells or as a basement membrane) and its various components (growth factors and proteins).

Importance of Three Dimensions

Culturing cells in a 3D environment promotes normal cell polarity, as opposed to a 2D culture in which cells are exposed to different upper and lower microenvironments, which results in an artificial cell polarity [26]. Growth on 3D substrates is not curtailed to a single plane. Hence the surface area over which cells can adhere and cell–cell communication can take place is enhanced. Because of the proximity of the cells to the ECM, the local concentration of important cytokines and enzymes may be greater than that distributed over a cell monolayer. Moreover, the presentation of ECM ligands to spatially oriented cells may afford the simultaneous stimulation of several signal transduction pathways [146], leading to greater control over cell behavior and function. Thus, 3D systems provide spatial regulation of the cells and also affect cellular responses to the physical and biochemical cues provided [147]. This may also lead to the remodeling of the ECM itself, thus modulating the dynamic reciprocity of the cell–ECM system more effectively. The significance of 3D culture versus 2D has been highlighted in many studies [148,149].

Substrates for Three-Dimensional Culture

It has been demonstrated that experiments carried out on a planar, rigid substrate elicit results that may not be comparable to those obtained under in vivo conditions [146]. To obtain a more biomimicking in vitro microenvironment, sufficiently porous substrates are increasingly being developed that provide spatial freedom to allow the movement of cells as well as the transport of nutrients. In addition, cell adhesion ligands and growth factors are being incorporated to bestow adhesive and proliferative properties to these substrates to recreate the natural environment. Substrates that serve as 3D environments include polymer scaffolds, hydrogels, porous cellulose beads, electrospun scaffolds [150], 3D printed scaffolds [151] and decellularized tissue [152].

Polymers for Three-Dimensional Scaffolds

Several types of synthetic polymers and naturally occurring biopolymers have been used to grow 3D tissues. Commonly used synthetic polymers include PEG, PLLA, poly(acrylic acid), and poly(lactic-*co*-glycolic acid) (PLGA). Synthetic polymers offer several advantages over naturally occurring biopolymers: they can be chemically defined and they are highly reproducible and xeno-free. They also offer opportunities for versatile chemical modifications and the tailoring of functional groups to facilitate the conjugation of different molecules of interest to the polymer backbone. However, synthetic materials have limited biodegradability and do not support the adhesion of all cell types. Short sequences of self-assembling peptides have been synthesized to mimic ECM proteins [153]. Naturally occurring biopolymers can also be used to fabricate scaffolds for 3D cell culture. Traditionally, collagen is the mostly commonly used 3D culture scaffold among biopolymer materials because it offers easy cell encapsulation and forms a hydrogel at 37°C in a gentle environment that does not damage the encapsulated cells. Alginate, gelatin, chitosan, gellan gum, and hyaluronic acid are among other naturally derived materials that have been used successfully for 3D cell culture [154].

These materials can be fabricated into 3D cell culture scaffolds by employing a variety of methods, depending on the requirements of specific tissue growth. Commonly used 3D scaffold fabrication methods are discussed next.

Preparation of Three-Dimensional Scaffolds

Hydrogel Scaffolds

Hydrogels are among the most commonly used material-processing techniques for 3D scaffold preparation and consist of a network of cross-linked hydrophilic polymer chains that can absorb water. Both synthetic and natural biopolymer materials can be fabricated into hydrogels that bear close resemblance to the tissues in terms of mechanical properties and water content [155]. Three important properties of hydrogels that influence 3D cell growth include the porosity, biodegradability, and source of the material. High porosity leads to more efficient cell infiltration and nutrient delivery to the inner sections of the 3D scaffold. If the hydrogel is biodegradable, it allows cells to remodel their microenvironment, which ultimately improves tissue growth. In general, bioactive materials (e.g., gelatin, collagen, and silk) promote cell adhesion, proliferation, and tissue growth versus synthetic material hydrogels (e.g., PEG hydrogel). However, they are poorly defined and inherent batch-to-batch variability confounds the

effects of the scaffold on cell functions. Hydrogels prepared with short, biodegradable, synthetic peptides that mimic extracellular proteins are increasingly being studied as fully defined 3D scaffolds [156].

Three-Dimensional Printed Scaffolds

3D printing involves the laying down of successive layers of material in additive manufacturing to form 3D shapes, which enables better control of pore morphology, size, and matrix porosity compared with other fabrication methods. With the help of computer-aided design and by using patient medical imaging data, 3D-printed scaffolds can be developed with an intricate design that are patient-specific. However, only a limited number of materials can be processed in 3D printers. Traditionally, 3D printing has been used to print molten materials such as metals, ceramics, and plastics. To fabricate scaffolds for tissue engineering, processes have been modified to print commonly used tissue engineering scaffold materials such as PCL, PLGA, and PEG. The ink for 3D printing can be used in two different forms: with or without cells. Once the material is deposited, it can be cross-linked by different methods such as heat, UV light, or binder [157]. 3D printing with inks containing cell suspensions is particularly interesting owing to its ability to fabricate cell-seeded scaffolds in a single step. The material needs to meet a number of requirements to be suitable for the 3D printing of scaffolds. It should possess suitable rheological properties to enable the deposition of strands of various thicknesses. Second, after deposition, the material should be mechanically strong enough to hold its shape until cured. Finally, the swelling of the material should be known so as to accommodate changes in the size of the printed strands. Schuurman et al. studied the concentration of gelatin methacrylate (GelMA) precursor, UV dose, and the incorporation of viscosity-enhancer to determine the parameters for the successful fabrication of 3D-printed scaffolds of gelatin strands [151]. 3D bioprinting also permits the fabrication of tissues with multiple cell types. Duan et al. printed mechanically robust, living trileaflet heart valves that contained aortic valve leaflet interstitial cells as well as aortic root sinus smooth muscle cells using alginate/gelatin bioinks [158].

Electrospun/Nanofibrous Scaffold

Electrospinning has evolved as a promising technique for fabricating fibrous scaffolds for 3D culture. This method produces nonwoven materials with fiber diameters ranging from a few nanometers to over 1 μm. In a typical electrospinning process, a polymer solution or melt is forced through a needle by using an electrostatic field, resulting in the formation of a jet that breaks up to produce fibers of adjustable diameter. The fibers are collected on a grounded substrate. Electrospun nanofibers are similar to native ECM in terms of morphology, which has led to their popularity as tissue engineering scaffolds [150]. A wide range of scaffold materials can be processed into scaffolds by electrospinning. A few limitations associated with electrospinning include poor cell infiltration and migration as a result of the close packing of scaffold fibers, the toxicity of the residual solvent, and the low mechanical strength of the scaffolds for load-bearing applications. Several strategies have been used to produce electrospun scaffolds with large pore sizes. These include the fine-tuning of electrospinning parameters [159] using sacrificial materials as porogens [160] and electrospinning in wet media [161].

Decellularized Tissue

Synthetic 3D scaffolds are an improvement over 2D scaffolds because they provide an in vivo–like microenvironment for promoting cell functions and tissue growth. However, they are lacking in two important aspects. First, synthetic scaffolds do not recapitulate the complete ECM protein composition and ultrastructure of the microenvironment. Second, they do not contain blood vessels, which are essential for large organs such as the heart and liver. Without the presence of vasculature in large synthetic scaffolds, the delivery of nutrients to cells farther from the surface of scaffolds becomes difficult when the scaffold size approaches that of organs such as heart or liver. These limitations of synthetic scaffolds pioneered the use of decellularized tissues and organs as scaffolds for 3D cell culture.

The cell-free organ is actually a combination of blood vessels and ECM secreted and remodeled by the native cells. By using the decellularized tissue or organ as a scaffold, the benefit of nature's own platform can be manipulated to engineer a new organ. However, it is essential that the methods employed for cell removal preserve the delicate structure of the ECM while ensuring complete cell removal from the organ or tissue. The choice of chemical agent to be perfused into the tissue or organ for decellularization depends on several factors such as tissue or organ density (e.g., dermis versus adipose tissue), the number of cells in the tissue or organ (e.g., liver versus tendon), the lipid content (e.g., brain versus urinary bladder), and the thickness (e.g., dermis versus pericardium) [162]. Every cell removal agent and technique will alter the ECM composition and cause some degree of ultrastructure disruption; however, efforts must be taken to minimize these undesirable effects. For example, detergents are effective agents

for cell removal because they solubilize cell membranes; however, they can also dissociate proteins in the ECM. Another challenge is the complete removal of the cell removal agent from the decellularized organ, because residual traces can damage the seeded cells [152]. Decellularization using physical forces has also been studied. The freeze–thaw process is a promising strategy for lysing cells without the loss of ECM proteins. However, postprocessing is required to remove the lysed cells from the tissue or organ. Furthermore, freeze–thawing can produce minor alterations in the ultrastructure of ECM [163]. Flynn et al. used the freeze–thaw process successfully to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells [164].

Development of Three-Dimensional Culture and Materials

New Materials

Numerous smart materials have been designed that incorporate unique material properties that benefit the 2D as well as 3D cell culture. These materials include stimuli-responsive materials that respond to external stimuli such as temperature, light, or pH, bioresponsive materials, shape memory polymers, double-network or interpenetrating network hydrogels, self-assembled materials such as peptide hydrogels, and photodegradable materials with spatiotemporal control of material degradation (Table 27.2). pH-sensitive materials are polyelectrolytes that contain weak acidic or basic groups, which either accept or release protons in response to changes in pH. In one study, pH-sensitive hydrogel scaffolds improved cell function, which was attributed to their ability to swell or contract in response to pH changes that modulated oxygen transport and cell infiltration [165]. Lei et al. fabricated a fully synthetic thermoresponsive hydrogel for the 3D culture of hPSCs [166]. These thermoresponsive scaffolds made of poly(*N*-isopropylacrylamide)-*co*-poly(ethylene glycol) could support the long-term and serial expansion of multiple human pluripotent stem cell lines with a high expansion rate. Moreover, the same hydrogel scaffolds supported the directed differentiation of stem cells. In another study, magnetic field–responsive hydrogels were fabricated by impregnating alginate scaffolds with magnetic particles; these scaffolds were seeded with aortic endothelial cells [167]. The cells were able to reorganize into cellular vessel-like structures in magnetically stimulated scaffolds without supplementing growth factors. In contrast, the cells formed sheets or aggregates in the nonstimulated (control) scaffolds. Hybrid cross-linking can also impart new properties to materials. GelMA is a commonly used photocurable hydrogel that has shown promising results in numerous tissue engineering applications [168]. However, it lacks mechanical strength and has limited control over the biodegradation rate. Gelatin is known to gel physically at low temperatures. In a study, this property of gelatin was exploited and GelMA precursor solution was UV–cross-linked after it had gelled at a low temperature. This new material with hybrid cross-linking (termed GelMA+) showed more than eightfold improvement in material strength and greater control over the enzymatic biodegradation rate (Table 27.2) [169].

New Technology Development

Advances in 3D cell culture have been made possible by the concurrent development of technologies that aided several aspects of 3D tissue engineering, such as control of the 3D microenvironment, fabrication of scaffolds for 3D culture, imaging of cellular interactions with the 3D microenvironment, and the large-scale manufacture of cells [179]. For example, technologies such as 3D printing, electrospinning, polymer demixing, microencapsulation, and microfluidic 3D cell culture have enabled the development of 3D scaffolds with an ECM-mimetic microenvironment and a controlled pore size. 3D printing technology has made feasible the fabrication of vasculature within the scaffolds by using 3D-printed sacrificial sugar templates in the hydrogel scaffold [180].

Characterizing cellular interactions with the 3D environment remains challenging because high-resolution 3D imaging is more complicated than 2D imaging. New techniques are required to comprehend the complex cellular interactions fully in 3D. CLARITY is a technique that transforms 3D tissue into a 3D nanoporous hydrogel-hybridized form that is optically transparent and macromolecule-permeable [181]. Superresolved fluorescence microscopy is a set of imaging techniques that offer nanoscale resolution by bypassing the diffraction limit of light, which is not achievable with conventional light fluorescence microscopy. This technique can also be used to image the 3D morphology of nanoscopic cellular structures [182].

Microfluidic platforms have given rise to the rapid generation of cell-laden microspheres for 3D culture. Toh et al. used a microfluidic platform to culture hepatocytes in a 3D environment. This system was used to test dose-dependent drug toxicity in vitro and predict in vivo hepatotoxicity [183]. Headon et al. generated size-controlled, biofunctionalized microgels for cell encapsulation using a microfluidic platform with precise control over the sphere size [184]. Alginates can be cross-linked in a salt bath in an environment that is gentle to cells. Therefore, alginates

TABLE 27.2 Some Examples of Materials for Three-Dimensional Cell Culture and Tissue Engineering

Material Property		Example Materials	Application	References
	Stimulus			
Stimuli-responsive	pH	pH-sensitive monomer DMAEMA-HEMA	Enhanced angiogenesis and tissue remodeling	[165]
	pH + temperature	SMO-PCLA-PEG-PCLA-SMO block copolymer	Bone tissue engineering	[170]
	Stress	PEG-poly(isocyano peptide)	Critical stress-mediated control of stem cell commitment	[171]
	Magnetic field	Hydroxyapatite (HA)-collagen ferrogel	Bone tissue engineering	[172]
	Temperature	Hyaluronic acid-p(NIPAM)	Adipose tissue engineering	[173]
Bioresponsive	Enzyme	PEG-heparin	Nerve regeneration	[174]
	Glucose	Chitosan-HA	Adipose tissue	[175]
Shape memory materials		Alginate-based Cryogel scaffold	Injectable hydrogels for cell transplantation	[176]
Interpenetrating polymer network (IPN)/hybrid materials		IPN of dextran and gelatin	Vascular tissue engineering	[177]
Self-assembled materials		Fmoc-FF and Fmoc-RGD–based short peptide hydrogel	Three-dimensional culture of human dermal fibroblasts	[153]
Photodegradable materials		Photodegradable poly(ethylene glycol)	Chondrogenic differentiation of encapsulated stem cells	[178]

PEG, poly(ethylene glycol); *DMAEMA*, *N*′,*N*′-dimethylaminoethyl methacrylate; *HEMA*, 2-hydroxyethyl methacrylate; *SMO*, sulfamethazine oligomers; *PCLA*, poly(epsilon-caprolactone-co-lactide); *pNIPAM*, poly(N-isopropylacrylamide); *Fmoc-FF*, fluorenylmethoxycarbonyl-diphenylalanine; *Fmoc-RGD*, fluorenylmethoxycarbonyl-arginylglycylaspartic acid.

have commonly been used to microencapsulate cells for 3D cell culture. The cells can be mixed with alginate precursor and dispensed into the aqueous salt solution using needles or droplet microfluidic platforms [185]. 3D culture has been applied to the field of stem cell technology as well. Pluripotent stem cells can be differentiated into different cell types within a 3D cell culture scaffold [186]. In addition, stem cells can self-organize into 3D tissues that can self-renew and differentiate when appropriate media, cell culture substrates, and biochemical cues are employed. These are called organoids and mimic some of the functions of real organs with applications as model organs for drug testing and other uses [187].

Finally, bioreactors are increasingly being developed for large-scale 3D cell culture. Bioreactors can have several designs, such as rotating wall vessels, direct-perfusion systems, hollow fibers, and spinner flask bioreactors [188]. At the core of a bioreactor is usually a small cylindrical chamber composed of a polymer scaffold that supports cell adhesion and growth as well as nutrient delivery. The bioreactor needs to maintain high levels of humidity and oxygen for optimum cell growth. An overview of the 3D technology development for cell culture and tissue engineering is provided in Fig. 27.1.

Cellular Responses to Three-Dimensional Substrates

Most cells in the in vivo environment are surrounded by other cells and the ECM in the third dimension. 3D substrates provide a more physiologically relevant environment for cell growth and tissue development. Although 2D cell culture methods have been well-established for many cellular studies, 2D culture methods cannot fully recapitulate the natural 3D environment. Therefore, in vitro 3D cell culture tests have been proposed to provide more predictable and reliable analysis with more relevance to the corresponding in vivo responses [189,190].

Researchers have discovered striking differences in cell responses, such as cell adhesion and cell migration, between 2D and 3D microenvironments [148]. Studies of cell–substrate interactions in the 3D environment become increasingly important for tissue engineering and the development of in vitro drug-testing models [179].

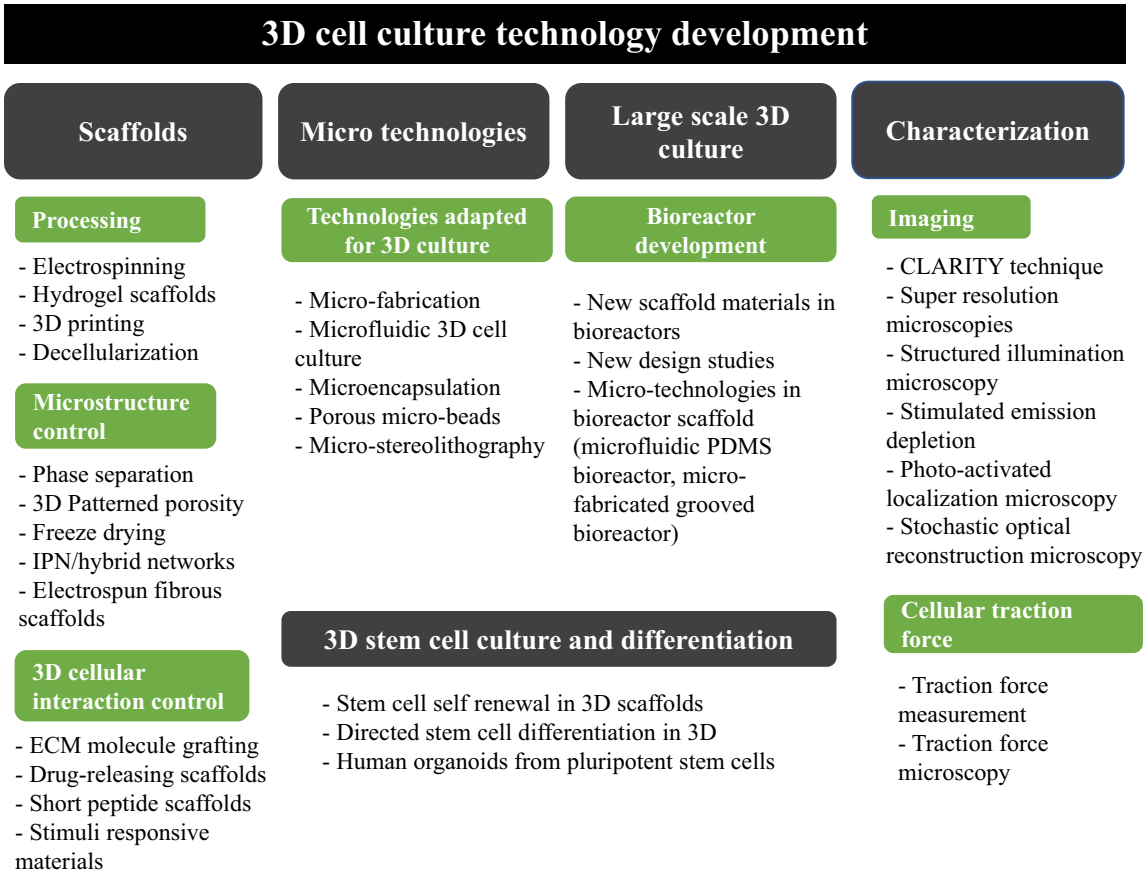

FIGURE 27.1 Overview of technology development for 3D cell culture tissue engineering. *3D*, three-dimensional; *ECM*, extracellular matrix; *IPN*, interpenetrating polymer network; *PDMS*, polydimethylsiloxane.

Cell Adhesion

Cell morphology is one of the major differences observed when comparing cells in 2D and 3D substrates [148,191]. On 2D substrates, cells are flat as either individual cells or a monolayer. The cells can adhere and spread in the horizontal x–y plane but they cannot spread in the vertical z dimension. Similar to cells on the basement membrane in vivo, the cells cultured on 2D surfaces can adapt an apicobasal polarity. The polarity and 2D cell morphology are relevant for some cell types, such as epithelial cells and endothelial cells. However, most mesenchymal cells assume a stellate morphology and polarize only during migration [192]. The geometries of 2D or 3D substrates also determined if integrin-mediated adhesions to the ECM form on only one side of the cell or on multiple planes around the cell surface. The difference in cellular responses with 2D substrates compared with 3D culture could arise from these variations in the spatial distribution of integrin–ECM adhesions [148]. Variations in matrix stiffness, structural diversity, and topography of the extracellular environment could affect the recruitment of proteins to adhesion sites, leading to the variations observed in different 3D contexts [193,194]. Although imaging FAs in 3D contexts is challenging because of the decreased size or intensity of adhesions, advances in imaging methods have enabled FA imaging in 3D matrices [195,196]. Thus, quantitative studies and models can be developed to provide insight into the mechanism of cell adhesion in 3D matrices.

Cell Migration

Advances in materials science, scaffold fabrication, biological tools, high-resolution imaging, and force traction measurement [197,198] have facilitated groundbreaking research in studying cell migration on 3D substrates. Studies on cell migration on 3D cell culture systems and in vivo conditions have revealed several differences compared with cell migration in 2D, including their morphology and mechanical and signaling control [148,199].

Using microphotopatterning lines, Doyle et al. showed that cell migration in both 1D and 3D is rapid and uniaxial, and the cell migration is independent of ECM ligand density but dependent on myosin II contractility and microtubules. The 1D and 3D migration were also characterized by an anterior microtubule bundle with a posterior centrosome [67]. Other researchers also illustrated the existence of multiple modes of 3D migration, including lamellipodial, lobopodial, and amoeboid migration, in which cells use different types of cell protrusions [200,201] and collective cell migration [202]. Another study showed that the classic polarized patterns of signaling, such as Rac and Cdc42, which guide migration in 2D models, are not essential for efficient 3D migration [199,202]. In contrast to lamellipodia-based migration on 2D, polarization signal molecules at the leading edge of 2D motile cells are not present in 3D lobopodia cell migration. Meanwhile, various studies showed that physical properties of the ECM such as stiffness, cross-linking, and pore size affect 3D migration [203,204]. Cells can also detect the porosity and the elasticity, linearity, or nonlinearity of the matrix, and adjust their migration mode accordingly [201].

Cell–Extracellular Matrix Reciprocity

3D culture also governs dynamic cell–ECM reciprocity, as seen in the response of cells to mechanical stress during matrix remodeling. In the case of fibroblasts grown on collagen matrices, this involves an increased density of collagen fibrils. As the fibroblasts exert a mechanical force on the underlying matrix, these collagen fibrils are either aligned or randomly oriented, depending on whether the matrix is restrained to the dish (scenario mimicking 2D culture) or free-floating, respectively. Alignment of the fibrils consequently affects cell morphology as stimulation with PDGF yields fibroblasts with a stellate morphology and isometric tension. In contrast, fibroblasts grown on the floating matrices (lack of tension in the matrix) adopt neuronal extensions and a dendritic network [204a].

The intrinsic state of the cell can also influence ECM adhesion by regulating integrin gene expression [205]. A study used a small interfering RNA-based screen of over 300 chromatin modifiers to identify functional interactions among different proteins that regulate differentiation of human epidermal stem cells [206]. One network of genetically interacting factors was found to affect distinct but functionally related genes, including those encoding α_6 and β_1 integrins and other proteins involved in mediating interactions with the ECM. The study showed that the chromatin modifiers that regulate differentiation are also involved by affecting genes encoding α_6 and β_1 integrins and other ECM-interacting protein, thus influencing stem cell interactions with the ECM. The study showed that epigenetic strategies are employed by the epidermal stem cells to protect from differentiation by adjusting cell–ECM adhesion.

Effect of Externally Applied Mechanical Stimuli

Cells reside in a dynamic environment in the body and are sensitive to changes in the microenvironment. Mechanical forces applied on the cell–polymer construct will often modify the cellular response, which renders cell–polymer interactions in the presence of mechanical stimuli an important area of study (Fig. 27.2).

Mechanical stimuli are particularly important in tissue engineering and regeneration, such as cardiovascular grafts, bone, cartilage, and tendon/ligament engineering, because different types of dynamic or static mechanical stresses are experienced by various tissues. The polymer substrate should possess physical properties that match the mechanical properties of the implant site. It should also have the ability to support the mechanical force exerted on the implanted graft at the site, such as pulsating blood flow through arteries and weight-bearing bone grafts.

In vitro systems have been developed to model the effect of mechanical stimuli in mechanical conditioning of the engineered tissues to enhance tissue functions. The biomimetic mechanical stimuli applied could be in the form of compressive, tensile, or shear force [207] (e.g., compressive stress on chondrocytes [208] or fluid shear stress on endothelial cells [209]). One type of experimental setup involves seeding cells on a flexible substrate such as an elastic membrane and applying a defined, stepwise, or cyclic strain to the substrate [210–212]. Bioreactors have also been designed to apply mechanical stimuli to induce functional maturation of the engineered tissue for tissue regeneration [213]. With the advancement of lab-on-chip technologies, microfluidic platforms have also been used to study how dynamic mechanical forces can affect cell–substrate interactions. Microfluidic chips have been designed to study various aspects of cell responses and to manipulate and analyze cells [214–216], cell migration [217], stem cell differentiation [218], and in vivo modeling [219].

The application of mechanical forces could trigger intracellular signaling via mechanosensors such as the actomyosin cytoskeleton and mechanosensitive ion channels [220,221]. The applied mechanical stimuli can also transduce signals to cells and hence direct stem cell differentiation via mechanosensing pathways such as RhoA signaling. For

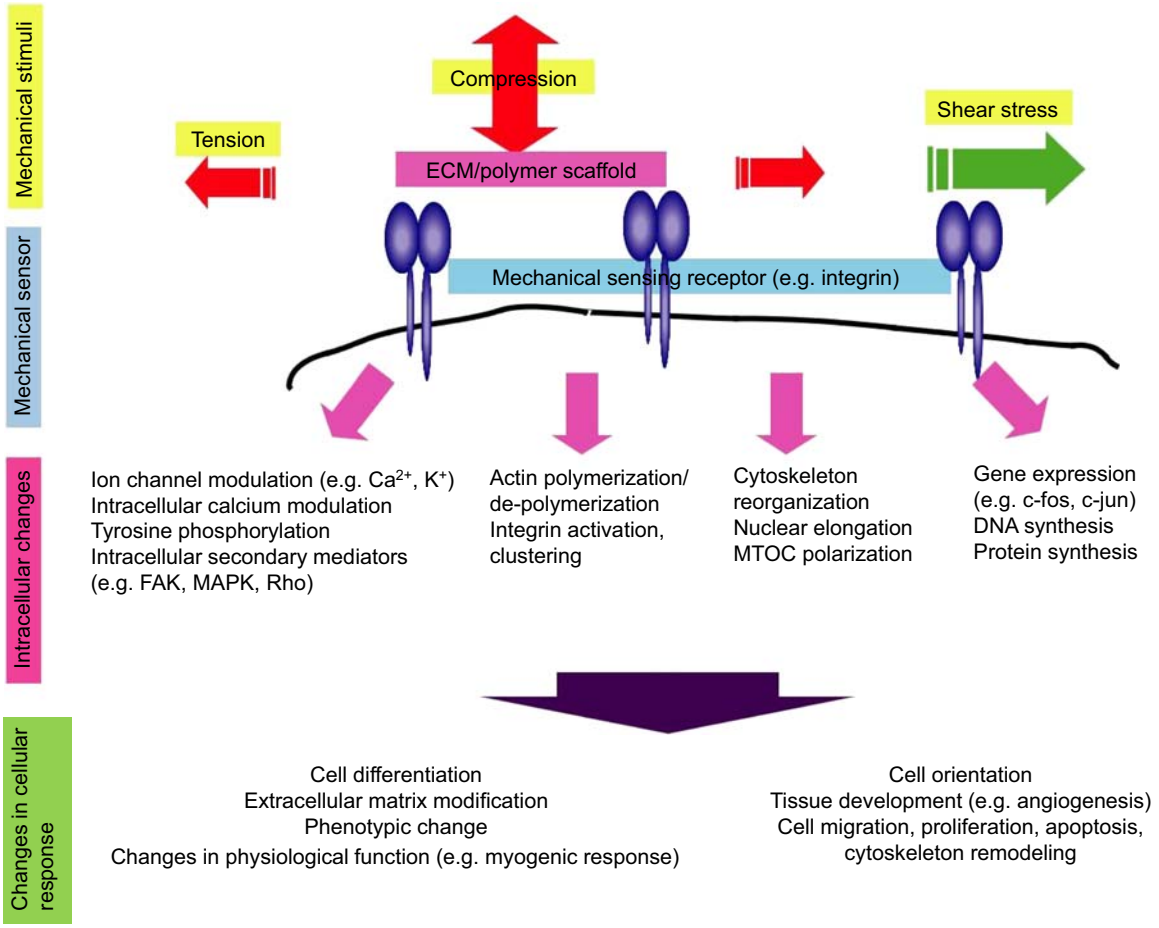

FIGURE 27.2 Effects of mechanical stress on cellular behavior. *ECM*, extracellular matrix; *FAK*, focal adhesion kinase; *MAPK*, microtubule-associated protein kinase; *MTOC*, microtubule organizing center.

example, when fluid flow is applied to C3H10T1/2 murine MSCs, RhoA and Rho kinase (ROCK)II are activated, which enhances Runx2 expression for commitment to osteogenesis [222].

Mechanotransduction

When a force is applied to cells growing on substrates, the cells sense the changes in the physical environment and transduce the mechanical signals to intracellular biochemical signals by a mechanism called mechanotransduction [223,224]. Mechanotransduction involves a series of complex and dynamic interactions between different cellular organelles and components [225] such as integrins, FAs, and the cytoskeleton, which collectively have important roles in biophysically induced cellular behaviors [225–227]. Various indirect and physicochemical mechanisms such as calcium signaling [228] and mechanosensitive ion channels [229] also contribute to mechanotransduction [92,207,230].

The class of integrin adhesion receptors is one of the cellular mechanosensors for mechanotransduction [231], as discussed previously. FAs are one of the most important integrin-mediated cell–matrix adhesions involved in mechanotransduction [225,232]. They are composed of a large complex network of adhesion molecules [233], including talin, vinculin, and FAK. FAs link the actin cytoskeleton to transmembrane integrins [11,234]; hence, the formation and maturation of FA are driven by cross-talk between both components [225].

FA mechanosensing has been shown to be crucial for mechanotransduction and force-mediated stem cell differentiation. FA maturation and actin polymerization of MSC were promoted in response to micropillar substrates [235]. The phosphorylation of FAK was also essential for topography-induced differentiation [124]. By inhibiting FAK activity, topography-induced differentiation was abolished. Various independent studies collectively demonstrated that FAK activity and cytoskeletal contractility have an essential role in topography-induced differentiation.

Force generation in the cytoskeleton is required for cell adhesion to ECM. The contractile cytoskeleton consists of actin, myosin, microtubules, and intermediate filaments. Increasing evidence has demonstrated the significance of the cytoskeleton in stem cell differentiation [123,125,236,237].

Contractile forces in nonmuscle cells are generated by nonmuscle myosin II. Cells use actomyosin contractility for a two-way interaction with the ECM. Engler et al. showed that matrices of different elasticity could regulate the differentiation of MSCs into different lineages [236]. The specific nonmuscle myosin II inhibitor blebbistatin blocked all elasticity-directed lineage specification without significantly affecting cell function and shape, thus providing evidence of cytoskeletal force generation in ECM sensing. This tension-mediated signaling is reflected by the reorganization of actin MFs or stress fibers to reflect surface features, such as their observed alignment to nanogratings [99,125,226,238]. This cellular force sensing also modulates Rho GTPase levels and MAP kinase activity for downstream stem cell gene regulation.

Rho GTPases are molecular switches that control various signal transduction pathways in cells [239] including the regulation of the actin cytoskeleton. Rho, Rac, and Cdc42 are the three best-characterized members of the Rho GTPase family. Many studies illustrate the importance of Rho in regulating the actin cytoskeleton in mechanotransduction. Increasing ECM stiffness or force application activates Rho [240], which, through a series of reactions, eventually promotes actomyosin stress fiber assembly [241], significantly changing the mechanical properties of the cell [242].

Using micropatterned islands of FN to control cell spreading, it appears that cell shape controls the osteogenic-adipogenic lineage commitment of MSCs through RhoA-dependent actomyosin contractility [237]. Adipogenesis is induced in MSCs confined to small ECM islands, whereas osteogenesis is induced in MSCs cells, with extensive spreading and higher cytoskeletal tension on large ECM islands. Inhibition of actin polymerization and tension by cytochalasin D or inhibition of ROCK activity mimics the phenotype of poorly spread cells, resulting in adipogenesis.

The intricate physical network described earlier sets the framework for the physical continuity spanning from the ECM to the nucleus. Mechanical signals such as topographical perturbations from the ECM can be transduced by structural alterations in the network to elicit differential gene expression in stem cells [243–245].

Whereas the actin MFs are anchored to the ECM through the integrins, they are also physically connected to the nuclear membrane in a coherent actomyosin cytoskeleton. Nesprins are a class of large outer-nuclear membrane proteins that bind actin MFs through their Klarsicht, ANC-1, Syne homology (KASH) domains [246,247], which are physically connected to the Sad1p, UNC-84 (SUN) domain protein of the inner nuclear membrane, forming a KASH–SUN complex to mechanically bridge the actin stress fibers to the nuclear membrane lamins. The nuclear lamina has an important role in mechanotransduction [248] and consists of a meshwork of intermediate filaments and lamin proteins that are physically associated with both the KASH–SUN complex [249,250] and the chromatin [245,251]. The linker of nucleoskeleton and cytoskeleton (LINC) complex, which includes the nuclear lamin and SUN binding to KASH domain of the actin-associated Nesprins, was shown to link both the nucleoskeleton and cytoskeleton. The presence of the LINC complex demonstrates that the mechanical forces arising owing to cell-substrate interactions and the substrate rigidity can physically affect the structural organization of the nucleus [246,252], potentially resulting in changes in gene expression. Forces that are transmitted to the nuclear scaffolds via the LINC complex may regulate critical DNA enzymes or factors. Indeed, the direct physical connection between the cell nucleus and cell–substrate interactions was illustrated in an earlier study [253]; the disruption of intermediate filaments leads to the mechanical decoupling of the integrins and nuclei.

Cellular Responses in Modifying Extracellular Matrix

The ECM forms the underlying substrate for cell adhesion, growth, differentiation, and mechanical support. It is known that connective tissue cells adapt their ECM to changes in mechanical load, such as in bone remodeling, or wound healing. In turn, changes in the ECM content can influence the performance of the tissue, such as the stiffness of heart and vasculature and the strength of bone and cartilage. Experimental evidence confirms that specific ECM proteins are regulated by mechanical stimuli in vivo. For example, tenascin-C and collagen XII are two ECM components associated with fibrillar collagen in tissues bearing high tensile stress such as tendons, ligament, periosteum, arterial smooth muscle, and heart valves [254–256].

In cardiac tissue, fibroblasts are the principal cell type responsible for secreting components of the ECM. In vitro studies using rat cardiac fibroblasts have shown long-term ECM component changes in response to variations in mechanical load. In response to both cyclic and static uniaxial stretch, an increase was obtained in both collagen I

and collagen II messenger RNA expression [257]. When the rat cardiac fibroblasts were cyclically stretched for various durations, mitogen-activated protein kinase was most rapidly activated and collagen I expression became most abundant [258].

CONCLUSION

Cell–substrate interactions are centrally important to many biological processes and have been investigated extensively from various angles. Discussed in the context of tissue engineering and regenerative medicine, this chapter covers the basics and general principles of this tremendously complex phenomenon. The examples cited here reiterate the importance and relevance of elucidating the interactions of cells with substrates (Fig. 27.3). The aim of this review is to provide a starting point for readers to design optimal substrates for specific tissue development. Despite advanced developments in fabrication and analytical technologies, many challenges remain for a deeper understanding of the cell–substrate phenomenon. The heterogeneous nature of the cell population in any tissue remains a challenge. Technologies for single-cell analysis and high-throughput assays have provided much knowledge. As the quality and quantity of the data improve with new characterization techniques, automated image analysis, efficient data analysis, and data mining of the massive data generated from high-throughput assays and computational modeling may become possible for developing a meaningful theoretical framework to describe and predict these cell–substrate interaction phenomena. The ability to fabricate a biomimicking environment to analyze cell–substrate interactions at the single-cell level in situ will also provide important insight: for instance, in uncoupling the effects of cell–cell communication from cell–substrate interactions. Finally, as the field of regenerative medicine continues to be fueled by advances in stem cell biology, studies of cell–substrate interactions, particularly with stem cells, transdifferentiation, and iPSCs, will be more important and rewarding in understanding tissue regeneration and disease development.

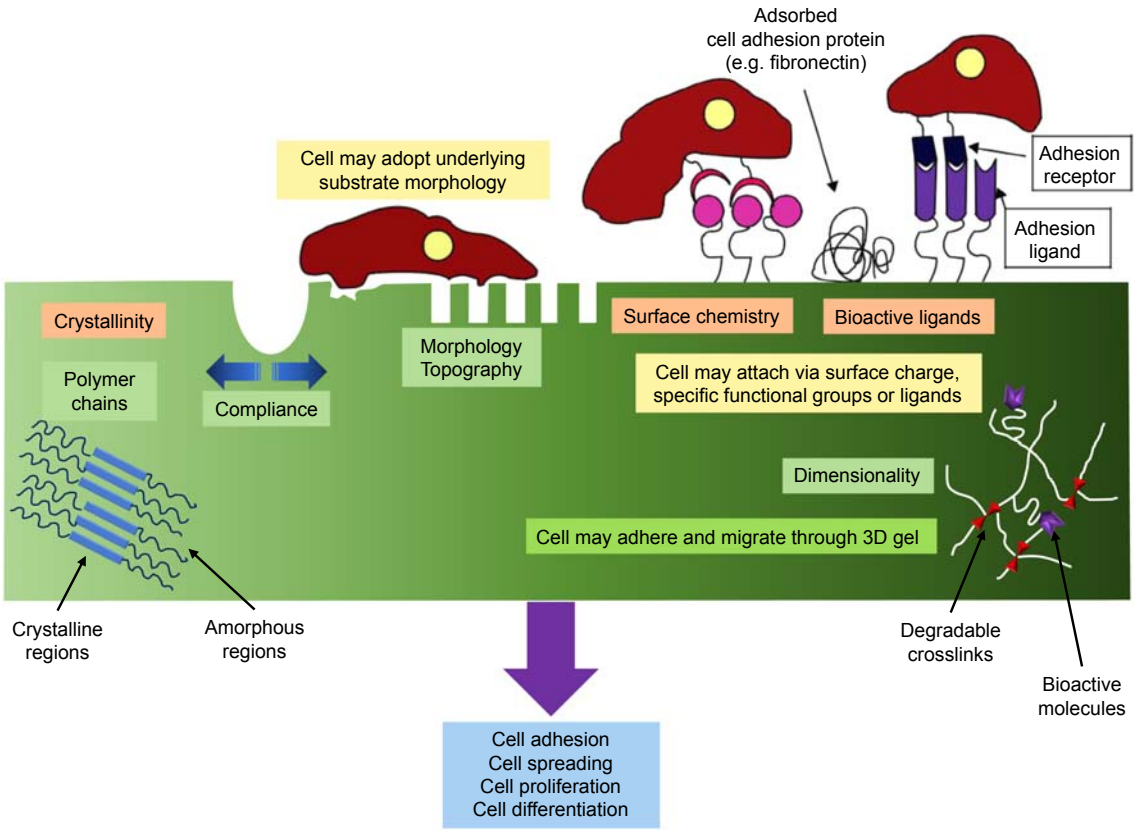

FIGURE 27.3 Schematic depicting the different properties of a substrate that influence cellular behavior. *3D*, three-dimensional.

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CHAPTER

28

Intelligent Surfaces for Cell Sheet Engineering

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INTRODUCTION

Cell- and tissue-based therapy has become a promising approach to curing a number of diseases and disorders. To obtain sufficient therapeutic effects, transplanted cell populations need to survive and then function appropriately in the transplanted site. The conventional method delivers cells directly by injecting suspended cells to the target site in the body; however, poor cell retention and survival at the target site are severe problems that prevent significant therapeutic effects on the damaged tissues. Tissue engineering, which was first proposed in the 1980s by R. Langer and J. P. Vacanti, promises to overcome this limitation in the field of cell-based therapy [1–3]. Delivery of cultured cells to damaged tissue in patients requires supporting materials for the primary cells that provide specific environments for cell growth in vitro while maintaining their structure during delivery. Advances in tissue engineering have produced scaffolds of various kinds of natural and synthetic polymeric materials to provide cells with a three-dimensional (3D) environment for adhesion, proliferation, and differentiation into a specific cell phenotype [4–6]. This technology is able to organize and arrange cells 3D in vitro. For example, collagen, fibrin, and alginate have often been used as a scaffold [7,8]. However, the implantation of naturally derived polymers may trigger an immune rejection in some patients, which has led to the wide use of synthetic polymers for tissue construction. For example, poly(lactic-*co*-glycolic acid) and its derivatives are commonly used for scaffold fabrication because their physical properties and degradation rates can be custom designed [9,10]. On the other hand, regardless of the scaffold materials used, most still have limitations such as insufficient cell migration into the scaffold and poor permeability to permit the ingress of cells and nutrients. In addition, the degradation of the scaffold can decrease the in vivo stability of scaffold-based tissues after implantation. Furthermore, in many cases, 3D scaffolds occupy some space within the constructed tissues that prevents cell–cell interaction. To overcome these limitations, a scaffold-free tissue generation system was needed for a new class of regenerative medicine technology. Intelligent thermoresponsive cell culture substrates can be used as powerful tools to establish a unique type of tissue engineering called "cell sheet engineering."

THE INTELLIGENCE OF THERMORESPONSIVE POLYMERS FOR CELL SHEET ENGINEERING

Thermoresponsive Polymer for Biomedical Applications

A number of stimulus-responsive polymers have been used as functional biomaterials in the fields of diagnostics, drug delivery system, and tissue engineering [11–14]. The physical properties of these materials change depending on the temperature or pH. In some other cases, light irradiation or exposure to an electrical and magnetic field can be used as a trigger to change their properties. Thermoresponsive poly(*N*-isopropylacrylamide) (PIPAAm) is well-known as a functional polymer for such uses as thermally induced drug release and thermally regulated separation systems [15–18]. The physical properties of PIPAAm change across its lower critical solution temperature (LCST) of

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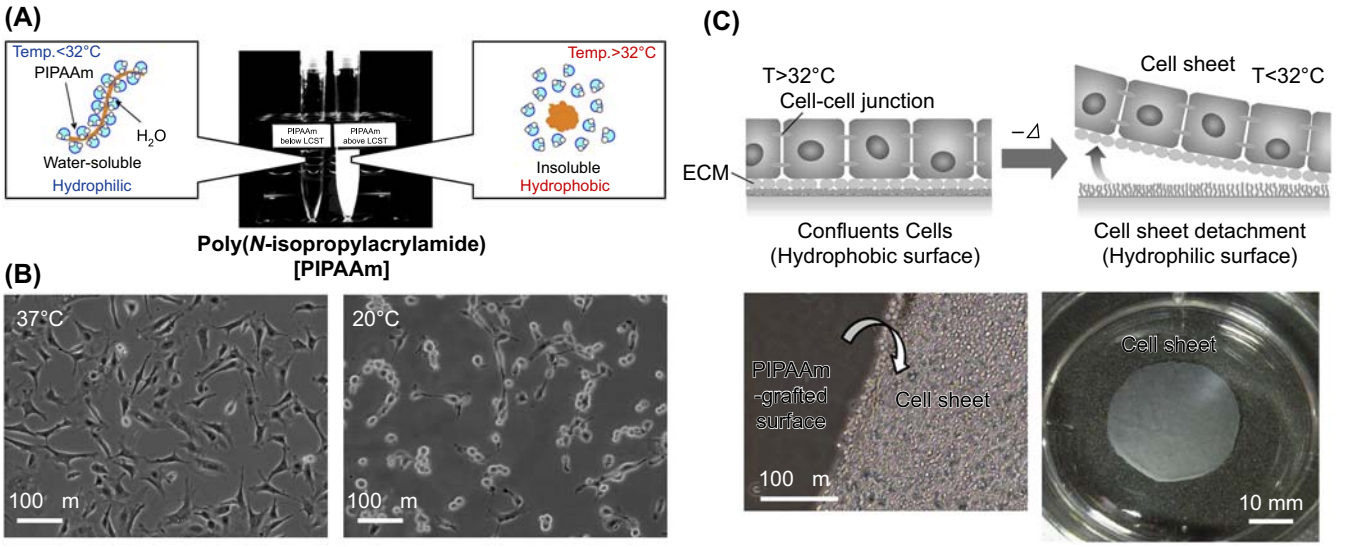

FIGURE 28.1 (A) Temperature-dependent change of hydrophilicity/hydrophobicity of poly(*N*-isopropylacrylamide) (PIPAAm) in solution. (B) Detachment of adhering cells on a thermoresponsive surface by lowering the culture temperature to 20°C. (C) Harvesting a cell sheet with preserved cell−cell junctions and associated extracellular matrix (ECM) without the use of enzymatic treatment. Photograph shows that the cell sheet shrinks two-dimensionally by detaching from the surface. *Adapted with permission from Takahashi H, Nakayama M, Yamato M, Okano T. Controlled chain length and graft density of thermoresponsive polymer brushes for optimizing cell sheet harvest. Biomacromolecules 2010;11:1991−99.*

32°C in aqueous media. Specifically, it has a hydrophilic property owing to the hydration of the polymer chain below the LCST, and then the chain conformation changes to a compact globule state above the LCST and the polymer becomes hydrophobic (Fig. 28.1A) [19]. This temperature-dependent switching behavior has been widely used in the field of drug delivery and allows us to design stimuli-induced release systems for various kinds of drugs (e.g., hydrophobic small molecules, biopharmaceuticals).

Thermoresponsive Surface for Regulating Cell Adhesion and Detachment

The thermoresponsive property of PIPAAm has been used to provide intelligence to a tissue culture substrate. PIPAAm-grafted surfaces behave as a cell adhesive surface at the normal culture temperature (37°C), whereas the surface becomes hydrophilic and they behave as a nonadhesive surface below the LCST. As a result, various kinds of cells adhering to the surface at 37°C detach spontaneously from the surface when the temperature is lowered below the LCST (for example, 20°C) (Fig. 28.1B) [20−22]. This thermally regulated cell detachment allows the adhered cells to be collected from the culture surface without an enzymatic treatment such as trypsinization. Importantly, when cells are confluent on the intelligent surface, a cellular monolayer can be harvested as a single continuous cell sheet by lowering the culture temperature (Fig. 28.1C). In conventional studies, naturally derived materials are widely used as scaffolds to provide an appropriate environment for transplanted cells and manipulate the therapeutic cells. On the other hand, the thermoresponsive surface is able to release a single cell sheet with only the associated extracellular matrix (ECM) (Fig. 28.1C). Moreover, because the cell sheet is harvested without enzymatic treatment, important membrane proteins and cell−cell junctions within the cell monolayer remain intact when it is transplanted. This unique tissue construction method is called "cell sheet engineering" and is applied to tissue engineering and regenerative medicine [23].

Controlled Grafting of Thermoresponsive Polymer on Culture Substrates

To achieve the regulation of cell adhesion and detachment, PIPAAm needs to be grafted precisely at a nanoscale. The amount grafted and its thickness are key factors to provide the appropriate hydrophobicity for cell adhesion at normal culture temperature; the surface is allowed to become nonadhesive by lowering the temperature across the LCST (approximately 30°C in culture medium). The PIPAAm is grafted by electron beam (EB) irradiation to an

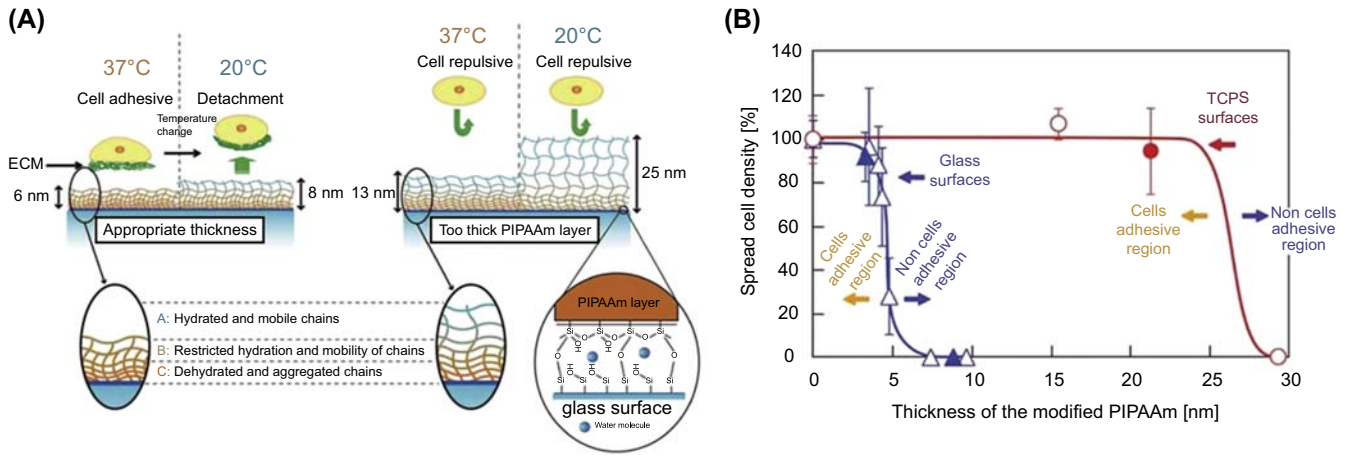

FIGURE 28.2 (A) Schematic illustration of molecular motion of poly(*N*-isopropylacrylamide) (PIPAAm) chains and cell adhesion and detachment behaviors on PIPAAm-grafted surfaces at different graft thicknesses. A PIPAAm layer at an appropriate thickness allows both cell adhesion at 37°C and cell detachment at 20°C. On the other hand, when the amount of PIPAAm is increased by changing electron beam irradiation, the surface is nonadhesive for cells regardless of the culture temperature. (B) Correlation of cell adhesion and thickness of the PIPAAm layer on a glass substrate and tissue culture polystyrene (TCPS) substrate. The range of the appropriate thickness is different between glass and TCPS substrates. *Adapted with permission from Fukumori K, Akiyama Y, Kumashiro Y, Kobayashi J, Yamato M, Sakai K, Okano T. Characterization of ultrathin temperature-responsive polymer layer and its polymer thickness dependency on cell attachment/detachment properties. Macromol Biosci 2010;10: 1117–29.*

N-isopropylacrylamide solution on a tissue culture polystyrene (TCPS) dish. As a result, PIPAAm can be grafted covalently and the amount grafted can be controlled at a nanoscale. The polymer thickness can be optimized by changing the monomer concentration and EB irradiation conditions. A previous study demonstrated that a PIPAAm layer approximately 20 nm thick is appropriate to achieve both cell adhesion and detachment by changing the temperature [25]. A thicker layer of PIPAAm makes the surface too hydrophilic for most types of cells, so they are unable to adhere in significant numbers on the surface even at a normal culture temperature (Fig. 28.2A). In addition, the appropriate thickness of the PIPAAm layer depends on culture substrates. For example, when PIPAAm is grafted on a glass substrate, a smaller amount of PIPAAm is optimal for regulating cell adhesion and detachment behavior owing to the difference in surface properties between TCPS and the glass surface (Fig. 28.2B) [26,27]. Therefore, to prepare a thermoresponsive surface for cell sheet engineering, precise control of the polymer grafting is required. Thermoresponsive culture dishes prepared using EB-irradiation methods are commercially available.

Variety of Fabrication Techniques of Thermoresponsive Cell Culture Substrate

Several kinds of polymer grafting techniques have been developed to produce thermoresponsive surfaces for cell sheet engineering. Surface-initiated living radical polymerization (LRP) processes can provide polymer brush–type thermoresponsive surfaces. Because the LRP processes are widely used to synthesize polymers at precisely controlled molecular weights, this polymer grafting is advantageous to fabricate well-defined thermoresponsive polymer brushes. Atom transfer radical polymerization and reversible addition-fragmentation chain transfer (RAFT) polymerization have been successfully used for PIPAAm brush grafting onto cell culture surfaces [24,28,29]. For example, because surface-initiated RAFT approaches allow the fabrication of polymer brushes with a uniform chain length, the grafted conditions can be precisely adjusted by controlling both the chain length and the graft density of the polymer (Fig. 28.3) [24]. On the other hand, a block copolymer of PIPAAm domain and a hydrophobic polymer domain can be used to achieve a simple coating on a culture surface by spin-coating the free polymer in solution [30]. In this approach, unlike the LRP process, PIPAAm can be synthesized as a free polymer, and then the culture surface can simply be coated with the polymers. To fabricate any kind of cell sheet, a suitable thermoresponsive surface needs to be selected according to the features of the cell sheet such as the cell type. This approach has promoted the development of a wide variety of thermoresponsive surfaces that have been used effectively to fabricate unique cell sheets.

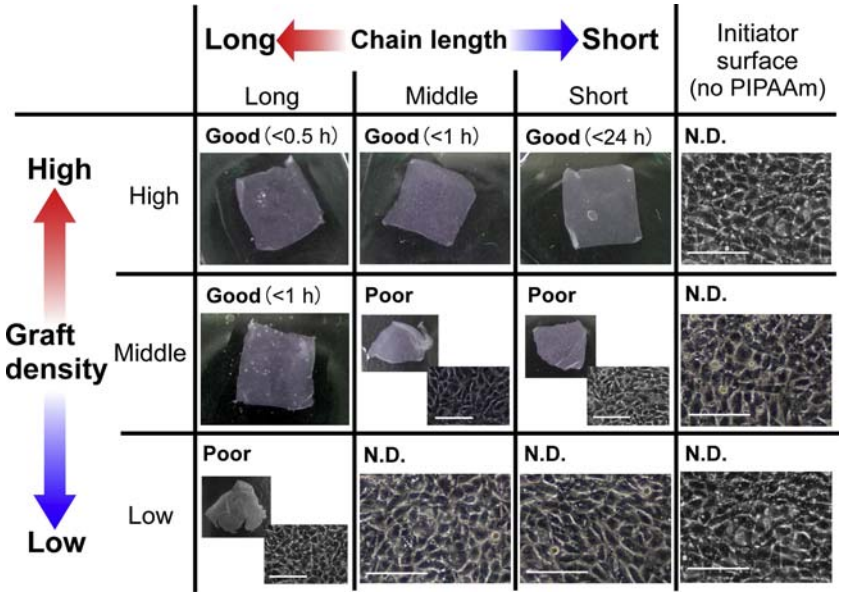

FIGURE 28.3 Performance of cell sheet harvesting using various kinds of poly(*N*-isopropylacrylamide) (PIPAAm) brush surfaces with different polymer chain lengths and graft densities. "Good" and "not detached" ("N.D.") in the photographs indicate complete cell sheet harvest by reducing the temperature (20°C) and no cell sheet detachment within 24 h by reducing the temperature, respectively. "Poor" indicates that some cell sheets showed complete harvest within 24 h but some did not. By changing the chain length and density of the PIPAAm brush (three different densities and chain length in the figure), thermoresponsive properties could be precisely controlled. Scale bar: 100 μm. *Adapted with permission from Takahashi H, Nakayama M, Yamato M, Okano T. Controlled chain length and graft density of thermoresponsive polymer brushes for optimizing cell sheet harvest. Biomacromolecules 2010;11:1991−99.*

CLINICAL APPLICATIONS FOR CELL SHEET ENGINEERING

The advantages of cell sheet engineering have been shown in a number of experimental studies as well as in human clinical studies of cell transplantation. The use of intelligent surfaces allows us to harvest therapeutic cells with complete preservation of cell−cell junctions and the associated ECM [31,32]. Therefore, a cell sheet can be manipulated simply without an artificial scaffold and transplanted just by placement onto a target site. Because the preserved ECM layer acts as a glue to adhere cell sheets tightly to the host tissue, this cell transplantation can be completed without additional treatments such as suturing. With these advantages, human clinical studies are ongoing targeting the regeneration of cornea, esophagus, heart, periodontal ligament, and cartilage.

Cornea Reconstruction

The unique cell sheet features mean that cell sheet engineering can be applied to treat epithelial tissues effectively. In fact, oral mucosal epithelial cell sheets have been exploited for corneal reconstruction. Severe trauma or eye diseases such as Stevens−Johnson syndrome can cause the complete loss of corneal epithelial stem cells and may result in corneal opacification with severe visual loss; to date no effective therapy has been established without limbal allograft transplantation. Cultured epithelial cell sheets derived from oral mucosal tissue have been successfully fabricated on the intelligent surface and are transplantable simply by handling with the support of a poly(vinylidene fluoride) membrane and placing the sheet onto the patient's corneal stroma without a scaffold [33]. The transplanted cell sheets adhere rapidly to the host corneal surface without suturing. A preclinical study using a rabbit model demonstrated that this cell sheet retained the corneal epithelium-like structure. After transplantation, it was confirmed that vascularization and inflammation were inhibited in the transplanted tissue and the corneal clarity and smoothness recovered significantly (Fig. 28.4). Clinical studies demonstrated that by transplanting the autologous epithelial cell sheet to human patients, this treatment with cell sheets provided good tolerability and efficacy after grafting.

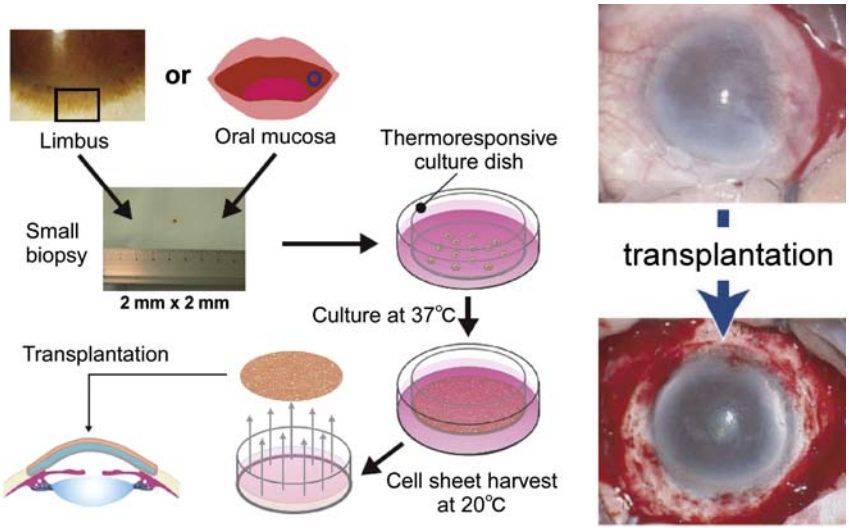

FIGURE 28.4 Schematic illustration of the preparation method for an oral mucosal epithelial cell sheet using the intelligent surface, and photograph of a transplanted cell sheet onto a diseased eye.

Esophagus Reconstruction After Endoscopic Submucosal Dissection Treatment

Mucosal epithelial cell sheets can be transplanted to an ulcer wound bed after endoscopic submucosal dissection (ESD). ESD is a commonly used operative procedure to resect superficial early-stage cancers in the gastrointestinal tract with low invasiveness compared with open surgery. However, postoperative inflammation and stenosis are still major complications observed in the resected area. Severe esophageal stricture after endoscopic treatment often requires repeated endoscopic balloon dilations or a temporary stent. Transplantation of autologous oral mucosal epithelial cell sheets promotes epithelialization of the resected area and prevents postoperative complications including esophageal stenosis [34–37]. These transplantable epithelial cell sheets retain all of the cell membrane proteins and ECM deposited during culture. Therefore, these cell sheets can easily adhere to and integrate into the transplanted tissue sites within a short period. In addition, the placement of cell sheets into the mucosal defect does not require sutures or other adhesives. Furthermore, an autologous epidermal cell sheet is useful for preventing esophageal stenosis after ESD. More autologous cell sheets can be fabricated than ones originating from oral mucosal tissue. Based on these animal studies, human autologous oral mucosal epithelial cell sheets are fabricated using an intelligent surface. Preliminary results showed early reepithelialization of the ulcer site; results suggest its efficacy for preventing stricture in cases of circumferential ESD. A further randomized study is being prepared to fully assess the potential benefits of using cultured autologous cell sheets therapies in regenerative medicine.

Myocardium Regeneration

Cell-based therapy is also predicted to be an important treatment for patients with severe heart failure including dilated cardiomyopathy (DCM) and ischemic heart disease. Skeletal myoblasts have been widely used in the clinical application of cell transplantation because of possible advantages including their autologous origin, high obtainability, high proliferative potential in vitro, and strong resistance to hypoxia followed by ischemia [38,39]. However, because of the poor retention of suspended cells to the target cardiac tissue, in many cases multiple injections are required to deliver high numbers of cells. In addition, the needle injection itself is highly invasive and induces cell death at the injected area, resulting in a risk for myocardial infarction [40]. To overcome these issues, the skeletal myoblast sheet derived from medial vastus muscle has been developed as a patch for myocardial regeneration. Sawa and coworkers demonstrated that autologous skeletal myoblast sheets harvested from the intelligent surface could be used to treat severe DCM [41,42]. Using this effective cell delivery approach, large numbers of myoblasts can be transplanted onto a damaged site. The myoblast sheets potentially release therapeutic cytokines including stromal-derived factor-1, hepatocyte growth factor, and vascular endothelial growth factor (VEGF) and deliver them to a damaged tissue effectively [43]. Although conventional scaffold-based cell transplantation also allows delivery of therapeutic myoblasts, it is unable to form the kind of cell-dense microenvironment found in a cell sheet construct.

In addition, its inflexible bulky properties are unable to withstand the dynamic pulsation of the host myocardial tissue. Studies demonstrated that this scaffold-free tissue delivery system has been also used to deliver other cell types (e.g., cardiac stem cell, adipocyte) in cell sheets to treat myocardial infarction in mice [44,45]. These strategies also have the potential to become a novel drug delivery system for myocardial treatment. In a case report, autologous myoblast sheets were transplanted into the heart of a patient with DCM. This cell sheet transplantation dramatically improved cardiac functions, resulting in no symptoms attributable to heart failure, left ventricular assist device weaning, and eventual hospital discharge.

Periodontal Ligament Regeneration

Periodontitis is an inflammatory disease characterized by periodontal tissue destruction. It predominates as the main cause of tooth loss in adults. It is well-known that periodontal ligament (PDL) tissues include stem cell populations and possibly differentiate into alveolar bone and cementum. A clinical study using autologous PDL cell sheets was carried out and achieved periodontal regeneration [46,47]. To fabricate PDL cell sheets in this human clinical study, PDL tissue was obtained from an unnecessary tooth such as a wisdom tooth. To deliver a large number of cells, triple-layered cell sheets were generally implanted in the proximity of the infrabony defect. No adverse events were found in nine patients and the safety and efficacy of the cell sheet approach were confirmed [48].

Cartilage Regeneration

Regenerative products for articular cartilage are available for patients with osteochondral defects but repeated abrasion of an articulated surface causes symptoms of immovable joints and pain. This radical treatment for osteoarthritis has not yet been established; current treatments such as nonsteroidal antiinflammatory drugs and injection of hyaluronic acid are usually conducted to retard disease progression. Triple-layer chondrocyte sheets might be applicable as a curative treatment for this partial-thickness defect of articular cartilage [49]. A clinical study is ongoing in which autologous chondrocyte sheets are transplanted onto the lesion of early middle-stage osteoarthritis. This scaffold-free cell transplantation is anticipated to be effective in patients with full-thickness cartilage defects as well as those with partial thickness defects.

CELL SHEET ENGINEERING PRODUCES SCAFFOLD-FREE, THREE-DIMENSIONAL TISSUE CONSTRUCTS

Cell Sheet Layering Technique

Progress in induced pluripotent stem cell (iPS) technology worldwide has opened up an entirely new era of life science research [50–52]. In the field of tissue engineering and regenerative medicine, iPS technology provides an important key to achieving cell-based therapies with the patient's own cells. In addition, it is expected that human cell-based tissue models will be developed for drug discovery and biological studies because patient-specific cells can be used to produce a customized tissue model for a specific disease. To exploit this excellent source of cells for medical applications, more integrated tissue engineering technology is required. 3D scaffolds from natural and synthetic polymers have commonly been used to provide a 3D environment for cell adhesion, proliferation, and differentiation into specific cell phenotypes. On the other hand, cell sheet engineering allows the creation of scaffold-free 3D tissues by layering multiple cell sheets. By placing one cell sheet onto another, the layered cell sheets bond tightly because of the preserved ECM underneath each individual cell sheet (Fig. 28.5) [53,54]. The cells can communicate with each other both physically and biologically [55–58]. The combination of these characteristics in the cell sheet layering process allows us to engineer functional 3D tissues. For example, cardiomyocytes cultured on the intelligent surface can be harvested as a single continuous cell sheet and each cardiomyocyte sheet exhibits synchronized beating within the cells of the sheet. Furthermore, when multiple beating cardiomyocyte sheets are layered, beatings at different rates are further synchronized throughout the entire 3D myocardial tissue construct [55,56]. This indicates that just by layering each cell sheet atop each other, they can interact electrochemically

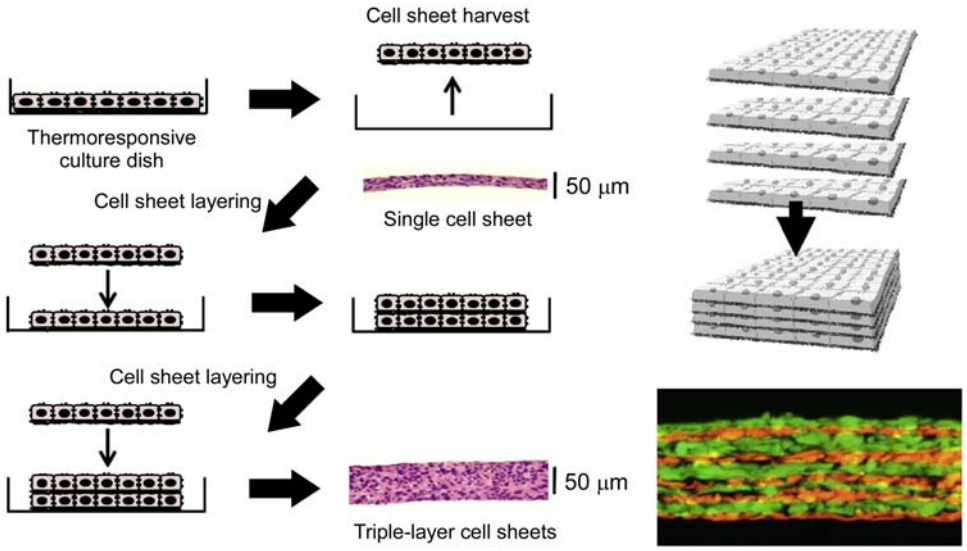

FIGURE 28.5 Cell sheet layering process for the production of a multilayer cell sheet construct without the use of scaffold. Because of the preserved extracellular matrix proteins, multiple cell sheets can be stacked tightly simply by layering them. *Adapted with permission from Haraguchi Y, Shimizu T, Sasagawa T, Sekine H, Sakaguchi K, Kikuchi T, et al. Fabrication of functional three-dimensional tissues by stacking cell sheets in vitro. Nat Protoc 2012;7: 850–8.*

between each cell layer. Therefore, this technique offers the potential for constructing large-scale 3D tissues without the use of a 3D scaffold.

Three-Dimensional Coculture System Based on Cell Sheet Layering

Cell sheet layering technology can be used to produce unique 3D cocultured tissue constructs. In the field of liver tissue engineering, because it is difficult to preserve hepatocyte-specific functions in vitro, several culture methods have been proposed, including coculture with other types of cells [59]. In particular, coculture methods with endothelial cells (ECs) significantly contributed to maintaining hepatic functions compared with conventional monolayer cultures [60,61]. However, the structural configuration of hepatocytes is considerably different from that of the natural liver. Cell sheet engineering can be applied to harvest a rat hepatocyte sheet from the intelligent surface and then sandwich it between two endothelial cell sheets (Fig. 28.6A) [62,63]. Within this 3D hepatic tissue construct, in vivo–like hepatocyte polarization was organized (Fig. 28.6B). This morphological similarity between the engineered rat hepatic tissues and native liver is advantageous for preparing bioartificial liver devices that require a highly effective delivery system for blood components to the hepatocytes. In addition, hepatocyte-specific functions, including albumin secretion, ammonia removal, and the induction of cytochrome P450, were highly preserved (Fig. 28.6C) [62]. The technology for layering multiple cell sheets is a simple operation and successfully reproduced both heterotypic/homotypic cell–cell and cell–matrix interactions with the native hepatocyte configurations. Because the 3D hepatic constructs closely mimic the in vivo environment, it could be valuable as a tissue model for drug screening, as an implantable tissue for cell-based therapies, and as an efficient culture platform for bioartificial liver devices [64].

Vascularization in Cell Sheets for Large-scale Tissue Construction

Although cell sheet layering is useful for producing large-scale tissues, most engineered tissues including multilayer cell sheets require vascularization to supply sufficient oxygen and nutrients inside the tissues [65–67]. As described earlier, synchronously pulsatile myocardial tissues have been successfully fabricated by stacking multiple cardiomyocyte sheets [55,56]. However, in a long-term in vitro culture, severe necrosis can be found within the

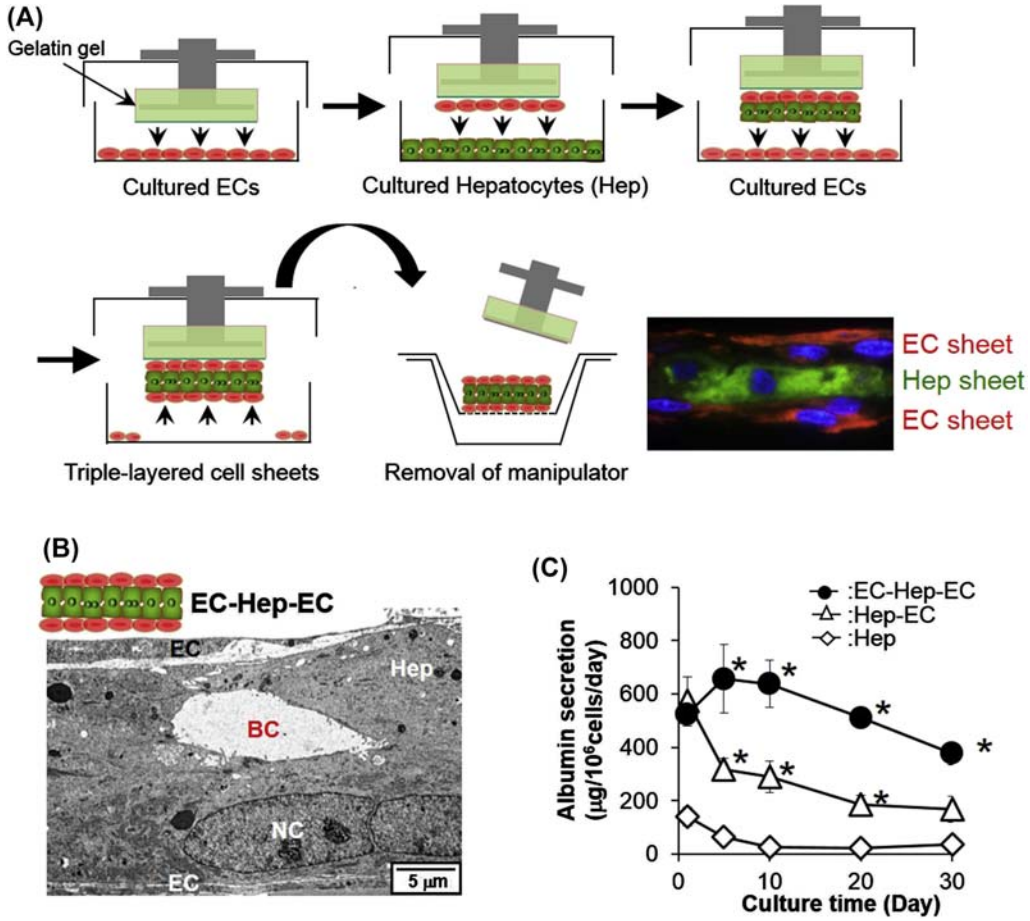

FIGURE 28.6 (A) Preparation of multilayer cell sheets composed of rat hepatocytes (Hep) and endothelial cell (ECs). (B) The formation of bile canaliculus (BC) can be observed only in multilayer cell sheets in which a hepatocyte sheet is sandwiched between two EC sheets (EC-Hep-EC). (C) Albumin secretion is maintained in the long term by sandwiching a hepatocyte sheet between two EC sheets, compared with double-layered hepatocyte and EC sheets and a single hepatocyte sheet. *NC*, nucleus. *Adapted with permission from Kim K, Utoh R, Ohashi K, Kikuchi T, Okano T. Fabrication of functional 3D hepatic tissues with polarized hepatocytes by stacking endothelial cell sheets in vitro. J Tissue Eng Regen Med 2017; 11: 2071–2080.*

engineered myocardial tissue [68]. This has been a limitation of producing thicker multilayer cell sheet constructs. Typically, in tissue transplantation, host-originated blood vessels invade the transplanted tissue, providing a supply of oxygen and nutrients within the tissue. However, thick tissues often become necrotic before sufficient neovascularization is established in the tissue. Moreover, neovascularization in engineered tissue cultured in vitro has not been achieved successfully. Coculture with EC is a promising approach to promoting vascular formation within engineered tissue. Through the cell sheet layering process, ECs can be incorporated simply by being sandwiched between two other cell sheets. Interestingly, the ECs form vascular-like branching networks in the layer cell sheets, which promote vascularization and connections to the host vasculatures after transplantation [69,70]. However, in an in vitro culture, maturation of the prevascularized networks is limited and perfusable tubular structures can rarely be found within the layered cell sheet construct. To overcome this problem and produce a thicker tissue, a technique for the in vitro formation of mature blood vessels was developed. To mimic in vivo conditions, layered cardiomyocyte sheets cocultured with ECs are transferred onto a vascular bed using a resected section of femoral tissue containing a connectable artery and vein [71,72]. For example, when a triple-layered cell sheet construct is placed on the vascular bed in the bioreactor system, the preserved ECM allows the cell sheets to adhere tightly onto the vascular bed; consequently, branching endothelial cell networks connect to the blood vessels originating from the vascular bed. After the formation of functional tubular blood vessels in the tissue construct, the cell sheets can survive owing to the newly formed vasculatures. This enables other triple-layered sheets construct to be layered onto the first cell sheet construct without causing necrosis, resulting in the production of six-layer cell sheets. Based

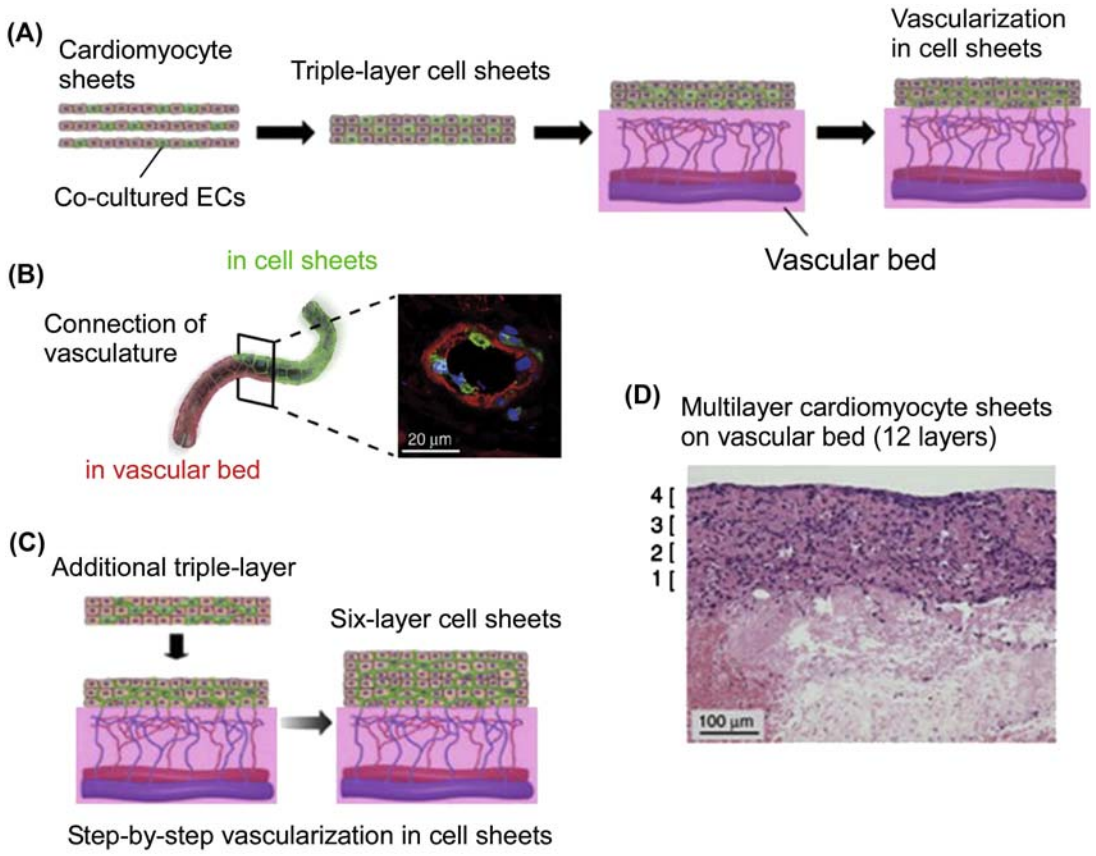

FIGURE 28.7 (A) Production of vascularized cell sheet constructs using a vascular bed. Endothelial cell (ECs) cocultured with cardiomyocyte sheets form a vascular-like branching network. (B) The EC network can connect to the vasculature in the vascular bed and oxygen and nutrients are supplied to the multilayer cell sheets through the newly formed vasculature. (C) After vascularization, step-by-step layering of additional triple-layer cell sheets produce thicker cell sheet constructs complete with new vasculature formations. (D) A previous study demonstrated that a 12-layer cardiomyocyte sheet construct could be produced using this vascular bed system without severe necrosis inside the construct. *Adapted with permission from Sekine H, Shimizu T, Sakaguchi K, Dobashi I, Wada M, Yamato M, et al. In vitro fabrication of functional three-dimensional tissues with perfusable blood vessels. Nat Commun 2013;4:1399.*

on this repeated cell sheet layering, the in vitro cell-dense tissues can be scaled up as desired. This technique may be applied to many other tissue types needed to generate thick vascularized tissues (Fig. 28.7).

COMBINATION OF CELL SHEET ENGINEERING AND SCAFFOLD-BASED ENGINEERING

Whereas cell sheet technology established scaffold-free tissue engineering, the unique properties of cell sheets could also be used jointly with scaffold-based tissue engineering. In the field of vascular tissue engineering, the basic strategy is to mimic a native blood vessel by combining vascular cells, mostly ECs and smooth muscle cells (SMCs), with a tubular scaffold under suitable culture conditions. Electrospinning is one of the most useful techniques for producing a tubular scaffold of synthetic polymers [73,74]. By seeding ECs onto a tubular scaffold composites of synthetic polycaprolactones and type I collagen, an engineered vascular tissue was produced and maintained structural integrity after implantation in a rabbit aortoiliac bypass model [75]. To produce mature blood vessels, SMCs have an important role because they contribute to the contractility and mechanical stability of mature vasculatures, and so they are often used as another cell source of vascular grafts [76,77]. However, because of the tubular-shaped geometry of the vascular scaffolds, cell seeding onto the exterior surface of the scaffold fails to achieve efficient cell penetration into the vascular scaffolds. In a cell sheet engineering approach, SMC sheets can be produced using a thermoresponsive substrate and the vascular graft can be effectively wrapped with the sheet

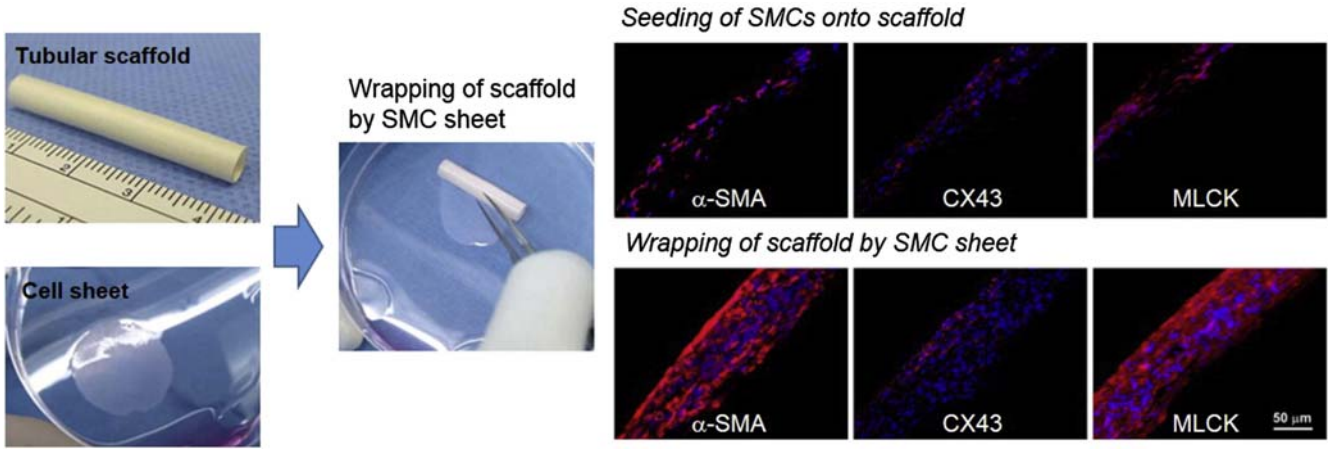

FIGURE 28.8 Fabrication of smooth muscle cell (SMC) layers on a vascular scaffold based on cell sheet engineering. By wrapping a tubular scaffold composed of polycaprolactone/collagen with SMC sheets, a mature smooth muscle layer can be efficiently produced, compared with one simply fabricated by seeding SMCs onto a scaffold. *α-SMA*, α-smooth muscle actin; *CX43*, connexin43; *MLCK*, myosin light chain kinase. *Adapted with permission from Ahn H, Ju YM, Takahashi H, Williams DF, Yoo JJ, Lee SJ, et al. Engineered small diameter vascular grafts by combining cell sheet engineering and electrospinning technology. Acta Biomater 2015;16:14–22.*

(Fig. 28.8) [78]. Cell sheet technology can produce a mature smooth muscle layer before it is applied to the vascular graft. Unlike simply seeding SMCs, the cells forming a cell sheet retain the all-important cell–cell junctions and express contractile proteins when they are applied onto the vascular scaffold. In addition, the secondary harvested cell sheet can be subsequently layered onto the first cell sheet and the graft can continue to be wrapped with multiple cell sheets. In a previous study, triple-layer SMC sheets were fabricated around a tubular scaffold. On the other hand, cell necrosis within the multilayer cell sheets needs to be addressed. In the previous study, a pulsatile perfusion bioreactor system was used to improve the nutrition supply and gas exchange. In addition, the pulsatile flow and pressure to the cell sheet–vascular scaffold led to tissue maturity that could withstand the level of blood flow from the native artery.

MICROFABRICATED INTELLIGENT SURFACE FOR ENGINEERING COMPLEX TISSUE CONSTRUCTS

Copatterning to Create a Cellular Microenvironment

Progress in cell sheet engineering technology has led to cell sheets being applied to the construction of various kinds of tissues and organs [79–82]. It has been confirmed that many different types of cells can be applied to fabricate cell sheets using a thermoresponsive surface. On the other hand, living tissues are composed of multiple cell types in which cell-to-cell interactions influence and maintain the development of characteristic physiological functions and activities. As described earlier, for example, ECs can provide the appropriate environment for hepatic cells under normal culture conditions [60,61]. In previous studies, it was demonstrated that when hepatic cells and ECs were copatterned on micropatterned surfaces, the patterned hepatocytes effectively maintained their functions even in 2D culture by optimizing the size of the hepatic cell patterns [83]. These results demonstrate that copatterned cell sheets of hepatocytes and ECs are one option for producing functional hepatic tissues. Micropatterning on a thermoresponsive surface supports the fabrication of copatterned cell sheets [84,85]. EB irradiation using photomasks onto a PIPAAm surface allows poly(*n*-butyl methacrylate) (PBMA) to be grafted spatio-selectively onto the surface (Fig. 28.9A). Because the LCST of the polymer-grafted regions is decreased by the presence of PBMA patterns, the regions become adhesive only at 27°C, whereas the nonirradiated regions show a cell-repellent property at the same temperature. Therefore, rat primary hepatocytes can be patterned on the PBMA regions at 27°C and then secondary seeded ECs can be patterned on the PIPAAm homopolymer regions at the normal culture temperature, resulting in the copatterning of hepatocytes and endothelial cells on the thermoresponsive surface (Fig. 28.9B–D). Because the copatterned hepatocytes, which can be harvested as a cell sheet (Fig. 28.9E), maintain the functions to synthesize albumin and urea, this type of functional hepatocyte assembly is expected to be useful for liver tissue engineering.

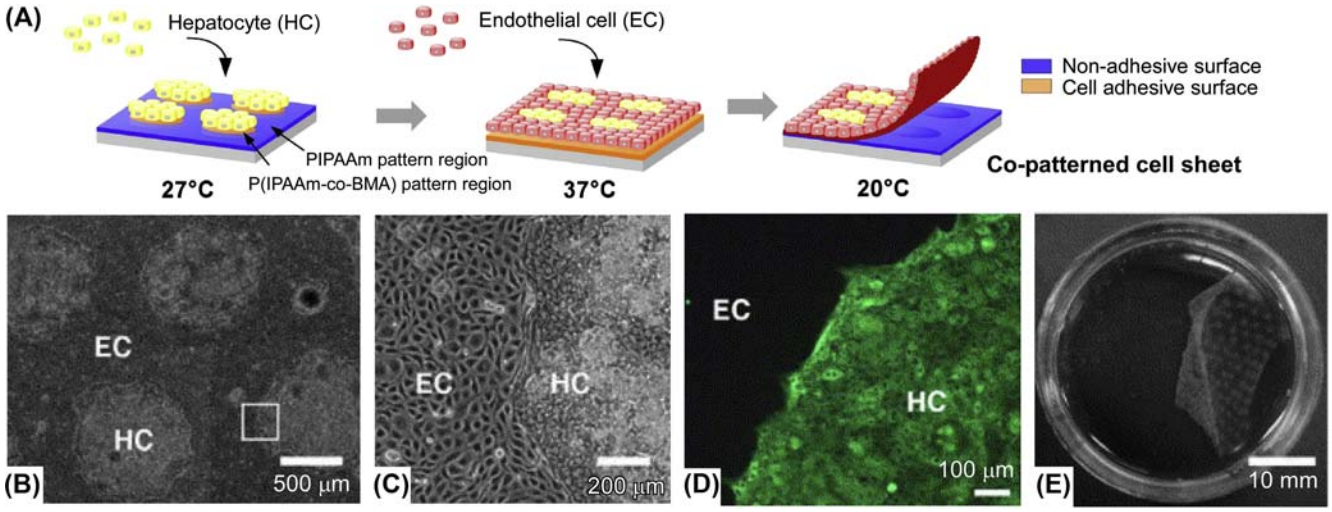

FIGURE 28.9 (A) Fabrication process of copatterns of rat primary hepatocytes (HCs) and endothelial cells (ECs) and harvest of a cell sheet using a micropatterned thermoresponsive surface. (B) Microscopic image of copatterned HCs and ECs. (C) Microscopic image in the square area in (B). (D) Fluorescence image of HC pattern on the thermoresponsive surface. (E) Photograph of a detaching cell sheet, composed of patterned HCs and ECs, by lowering the culture temperature. *P(IPAA-co-BMA)*, poly(isopropylacrylamide-co-butyl methacrylate); *PIPAAm*, poly(*N*-isopropylacrylamide). *Adapted with permission from Tsuda Y, Kikuchi A, Yamato M, Nakao A, Sakurai Y, Umezu M, et al. The use of patterned dual thermoresponsive surfaces for the collective recovery as co-cultured cell sheets. Biomaterials 2005;26:1885–93; Tsuda Y, Kikuchi A, Yamato M, Chen G, Okano T. Heterotypic cell interactions on a dually patterned surface. Biochem Biophys Res Commun 2006;348:937–44.*

Regulating Cell Orientation in Cell Sheet Engineering

Intelligent Surfaces for Regulating Cell Orientation

Native tissues often form specific structures that are well-known important factors for producing an appropriate functionality [86–89]. Ideally, engineered tissues should be produced in an environment that closely mimics the microstructure of native tissue. In particular, some kinds of native tissues, such as muscle tissues, have well-organized orientations of cells and/or ECM, and their tissue anisotropy are a critical factor in providing biomimetic functions to engineered tissues. For example, in native skeletal muscle, the bundle structure of highly oriented myofibers is essential for generating mechanical functions. Micropatterning approaches can be used to regulate the cell orientation on culture substrates [90]. Although a variety of microfabricated substrates have been reported, conventional substrates have limitations to releasing the well-organized cell monolayer from the culture surface. Because the intelligent surface allows the harvest of aligned cells as a single-cell sheet, cell sheet engineering overcomes these limitations to facilitate the design of 3D cell orientation within a densely packed cell environment. To achieve this, several kinds of micropatterned thermoresponsive substrates have been developed [91,92]. Using these substrates, cell orientation is first regulated 2D and then the aligned cells are stacked as cell sheets layer by layer. Microgrooved polydimethylsiloxane substrates are widely used to control cell alignment, and the same method can be applied to produce aligned cells on the thermoresponsive surface [93]. First, a microgrooved TCPS substrate is prepared and then PIPAAm is grafted over the whole surface. As a result, the surface functions to regulate cell orientation and release the cell sheet. The LRP process is also effective for producing micropatterns on a thermoresponsive polymer layer. Through two-step polymerization, hydrophilic poly(*N*-acryloylmorpholine) (PAcMo) can be grafted site-specifically on a PIPAAm layer, resulting in block polymer, PIPAAm-*b*-PAcMo domains and PIPAAm domains (Fig. 28.10A) [94,95]. The width of the patterns is a key factor in the regulation of cell orientation, and the previous study demonstrated that a 50 × 50-μm striped pattern was appropriate for producing a cell sheet composed of aligned cells. Because of the difference in the cell-to-surface affinity on the surfaces, cells are aligned parallel to the stripe patterns simply by cell seeding. For example, human dermal fibroblasts proliferate and reach confluency while maintaining alignment on the patterned surfaces (Fig. 28.10B) [92,95]. Harvested cell sheets typically shrink 2D and maintain the original aspect of the cell monolayer, whereas here the aligned actin fibers in the cell monolayer induced two distinctive shrinking rates in the vertical and parallel sides of the cell alignment (Fig. 28.10C). This suggests that the cell orientation influences the physical properties of cell sheets. In addition, the cell alignment induces a change in the biological properties of cell sheets. A previous study demonstrated that VEGF secretion from a normal human dermal fibroblast sheet was increased by organizing the alignment of fibroblasts [95]. As described

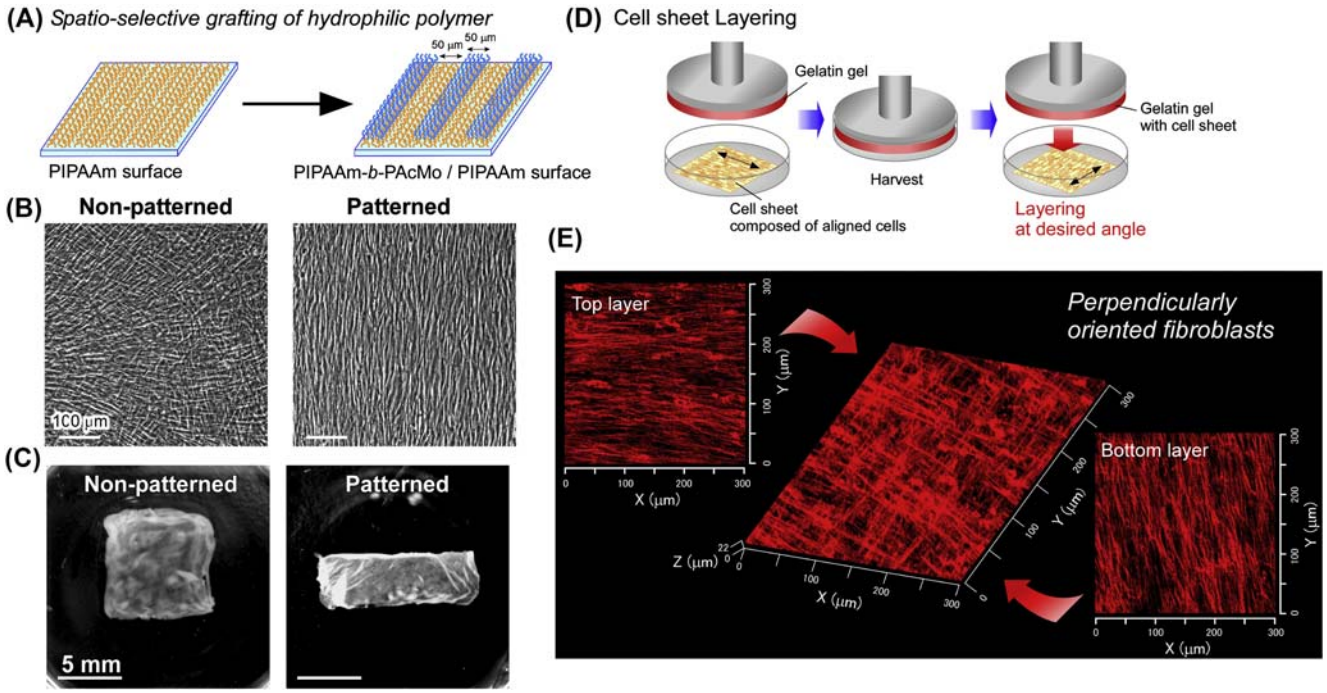

FIGURE 28.10 (A) Fabrication of micropatterned thermoresponsive surface composed of poly(*N*-isopropylacrylamide) (PIPAAm) regions and PIPAAm-*b*-poly(*N*-acryloylmorpholine) (PAcMo) regions through spatio-selective grafting of PAcMo on a PIPAAm-grafted surface. (B) Microscopic images of fibroblast sheets prepared using normal (nonpatterned) and patterned thermoresponsive surfaces. (C) Photographs of cell sheets harvested from normal (nonpatterned) and patterned thermoresponsive surfaces by lowering culture temperature. (D) Schematic illustration of layering process of cell sheets composed of aligned cells using a gelatin gel-coated manipulator. (E) Fluorescence image of perpendicularly layered fibroblast sheets produced by cell sheet layering. Actin fibers of cell sheets are stained red. *Adapted with permission from Takahashi H, Nakayama M, Shimizu T, Yamato M, Okano T. Anisotropic cell sheets for constructing three-dimensional tissue with well-organized cell orientation. Biomaterials 2011;32:8830–8.*

earlier, vascularization is one of the main issues to constructing large-scale tissue successfully [71,72] and angiogenic growth factors such as VEGF are important mediators to promoting vascularization in tissues. Therefore, this could potentially enhance vascularization in multilayered cell sheets. These patterned substrates are also able to regulate the orientation of osteoblasts, SMCs, and mesenchymal stem cells [91].

Arrangement of Three-Dimensional Orientation Using Cell Sheet Layering Techniques

Because cell sheets can be manipulated using a gelatin gel-coated plunger [53], cell sheets composed of aligned cells can be layered while maintaining the designed orientation. In some native tissues, tissue anisotropy is organized 3D and the orientation of their structures is essential for specific mechanical and biological functions. For example, native myocardial tissue consists of multiple layers of aligned cardiomyocytes that are oriented in various directions throughout the whole tissue [88,89,96]. This well-organized 3D oriented structure is important for generating the anisotropic electrical propagation found in the native myocardium. However, it remains difficult to arrange different cell orientations vertically using conventional biomaterial scaffolds. Employing the cell sheet layering technique, cell sheets composed of aligned cells can be layered upon each other to achieve the desired directions (Fig. 28.10D). For example, 3D perpendicularly oriented fibroblast sheet constructs can be created without using a 3D scaffold (Fig. 28.10E) [95]. This strategy is simple and flexible for creating a variety of tissue architectures. As described earlier, the cell-dense tissue structure is important for multiple cell sheets to communicate with each other within the layered cell sheet construct. Therefore, this technique of arranging cell orientation within engineered tissues is expected to be useful for producing biomimetic myocardial tissues.

Skeletal Muscle Tissue Engineering

Because native skeletal muscle tissue is made of highly oriented myofibers, the orientation of muscle cells needs to be regulated to produce biomimetic muscle tissue constructs [97–99]. Using the micropatterned intelligent surface, it is possible to align human skeletal muscle myoblasts in a direction parallel to the striped patterns (Fig. 28.11A) [57,58].

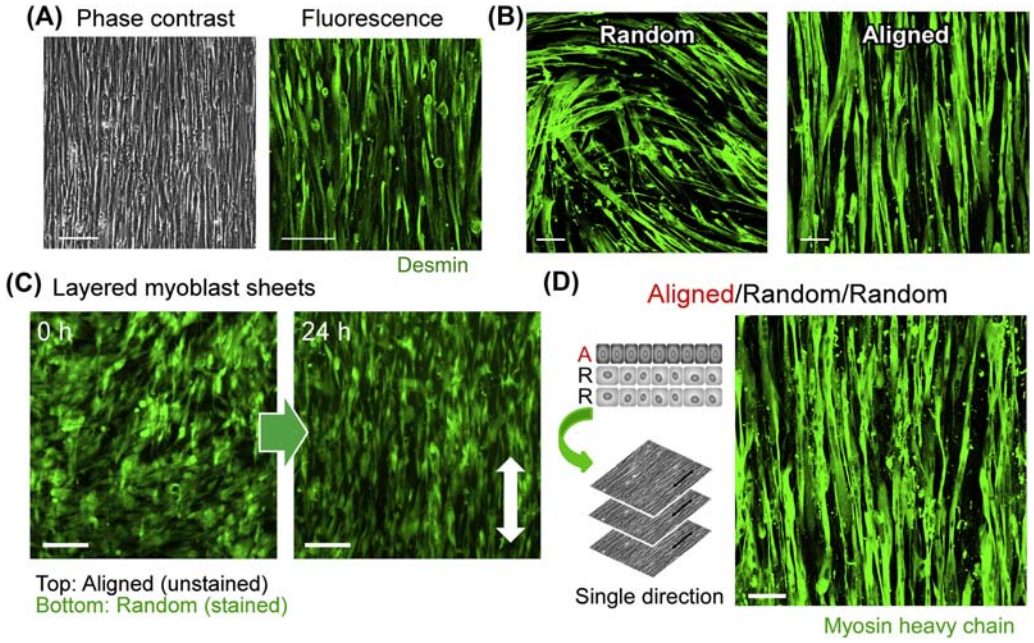

FIGURE 28.11 (A) Myoblast sheet prepared using a micropatterned thermoresponsive surface. (B) Myotubes differentiated from random and aligned myoblasts fabricated on normal and patterned surfaces. (C) Rearrangement of cell orientation of random myoblasts (bottom cells: fluorescently stained) by layering a cell sheet composed of aligned myoblasts (top cells: unstained). (D) Myotube construct produced through the differentiation of three myoblast sheets (top layer: aligned myoblasts; middle and bottom layers: random myoblasts). The random myoblasts self-organize their orientation with the top cell sheet's alignment after they are layered. *Adapted with permission from Takahashi H, Shimizu T, Nakayama M, Yamato M, Okano T. The use of anisotropic cell sheets to control orientation during the self-organization of 3D muscle tissue. Biomaterials 2013;34: 7372–80.*

After reaching confluence, the aligned myoblasts can be harvested as a single continuous cell sheet by lowering the culture temperature to 20°C. Then, after culturing the aligned myoblasts in differentiation media (e.g., 2% horse serum–containing media), they differentiate into myotubes while maintaining their aligned orientation (Fig. 28.11B). In addition, regulating the aligned orientation stimulates the formation of longer myotubes, compared with randomly oriented myoblasts. This aligned structure will be important for producing biomimetic muscle tissue [100]. Moreover, because the aligned myotubes can be layered with cell sheet technology, this combination supports the production of 3D aligned muscle tissues, which is required for scaling up skeletal muscle tissues. On the other hand, previous study found a unique behavior of myoblasts [57]. When a single cell sheet composed of aligned myoblasts was layered onto a normal cell sheet composed of randomly oriented myoblasts, the bottom myoblasts rearranged their orientation in the same direction as the top aligned cells (Fig. 28.11C). Consequently, all myoblasts became aligned within the layered cell sheet construct. Although the mechanism has not yet been elucidated, this self-organization process possibly shows an important factor for regulating cell–ECM orientation in native muscle tissues. Furthermore, this self-organizing behavior allows us simply to produce 3D myotube constructs with a single orientation. Because the top cell layer stimulates random cells to become aligned, three cell sheets of aligned myoblasts are not necessary to produce a triple-layer myotube construct with a single common direction. Myoblasts in the tissue construct are able to self-adjust their 3D orientation precisely. After self-organization is completed, the 3D tissue construct forms an aligned structure of myotubes (Fig. 28.11D). This cell sheet–based technology provides a new potential for constructing complex tissues composed of natively oriented cell assemblies, particularly for skeletal muscle tissue [58].

CONCLUSIONS

Progress in tissue engineering enables cells to be cultured 3D on polymeric scaffolds and then transplanted at desired sites for tissue regeneration. Scaffold-based tissue engineering continues to develop, but cell sheet engineering has also expanded its unique features and advantages to create a new field in regenerative medicine. Intelligent surfaces allowing us to use cell sheets have been applied to human clinical studies that deliver therapeutic cells to

damaged sites. Moreover, the cell sheet transplantation approach will probably be applied to other tissue regeneration applications in the near future. Moreover, new cell sheet technologies including polymer grafting, cell patterning, and cell sheet layering promise to advance the production of sophisticated 3D tissues with complex structures. To develop an integrated tissue model for future drug discovery, we need to develop techniques to construct more complex tissues. To meet this challenge, a new level of cell sheet engineering must be developed to be able to construct any complex tissue with well-organized structures. The combination of microfabricated and thermoresponsive substrates will provide anisotropy within the cell sheets and allow us to use them as "anisotropic tissue units" to produce flexible tissue constructs with multiple 3D orientations. Progress in cell sheet engineering has shown the potential to produce scaffold-free 3D oriented tissue constructs with complex microstructures mimicking native tissues.

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CHAPTER

29

Applications of Nanotechnology for Regenerative Medicine; Healing Tissues at the Nanoscale

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INTRODUCTION

Regenerative medicine aims to develop methods to regrow, repair, or replace damaged or diseased cells, tissues, or organs [1] based on integrated strategies from tissue engineering, molecular biology, biomaterials, and stem cell biology. Tissue regeneration can be achieved by stimulating the repair mechanisms of the body, particularly by the delivery of stem cells that can perform biological functions and materials that can function as scaffolds to promote cell proliferation. Because human tissues have a nanoscale structure (Fig. 29.1), progress in nanotechnology has accelerated advances in regenerative medicine with the ability to mimic the composition and structure of human tissues and organs at the nanoscale [4,5] (Fig. 29.2). In addition, many functional nanomaterials have been used to deliver drugs, proteins, and genes for tissue regeneration [4].

Since Richard Feynman first proposed the idea of developing technology at the atomic scale in 1959, nanotechnology has been rapidly developed and actively investigated in many biomedical research fields. According to the US National Nanotechnology Initiative, nanotechnology is defined as "the understanding and control of matter at dimensions of roughly 1−100 nm, where unique phenomena enable novel applications." Nanotechnologies have been incorporated into multiple research areas related to regenerative medicine, including drug delivery, stem cell therapy, and scaffolds for tissue engineering [6−9]. For example, thermoresponsive liposome (TSL) nanoparticles can release their cargo at the liposome transition temperature [10]. When doxorubicin (DOX) was loaded into TSL nanoparticles, only a small amount of leakage was monitored at 37°C. As the temperature was raised to 42°C, almost 100% of DOX was released from TSL nanoparticles. In this respect, TSL nanoparticles have the potential to be applied to target a tumor site where the temperature is higher (~40−42°C) compared with normal tissues (~37°C) [10].

In this chapter, we will present the unique physicochemical properties of nanomaterials for applications in regenerative medicine. In addition, we will discuss nanotechnology-based strategies in regenerative medicine for treating multiple types of tissues, including bone, muscle, blood vessels, and neural tissues. We will also introduce nanotechnology-based stem cell therapy, particularly for controlling stem cell fate and function.

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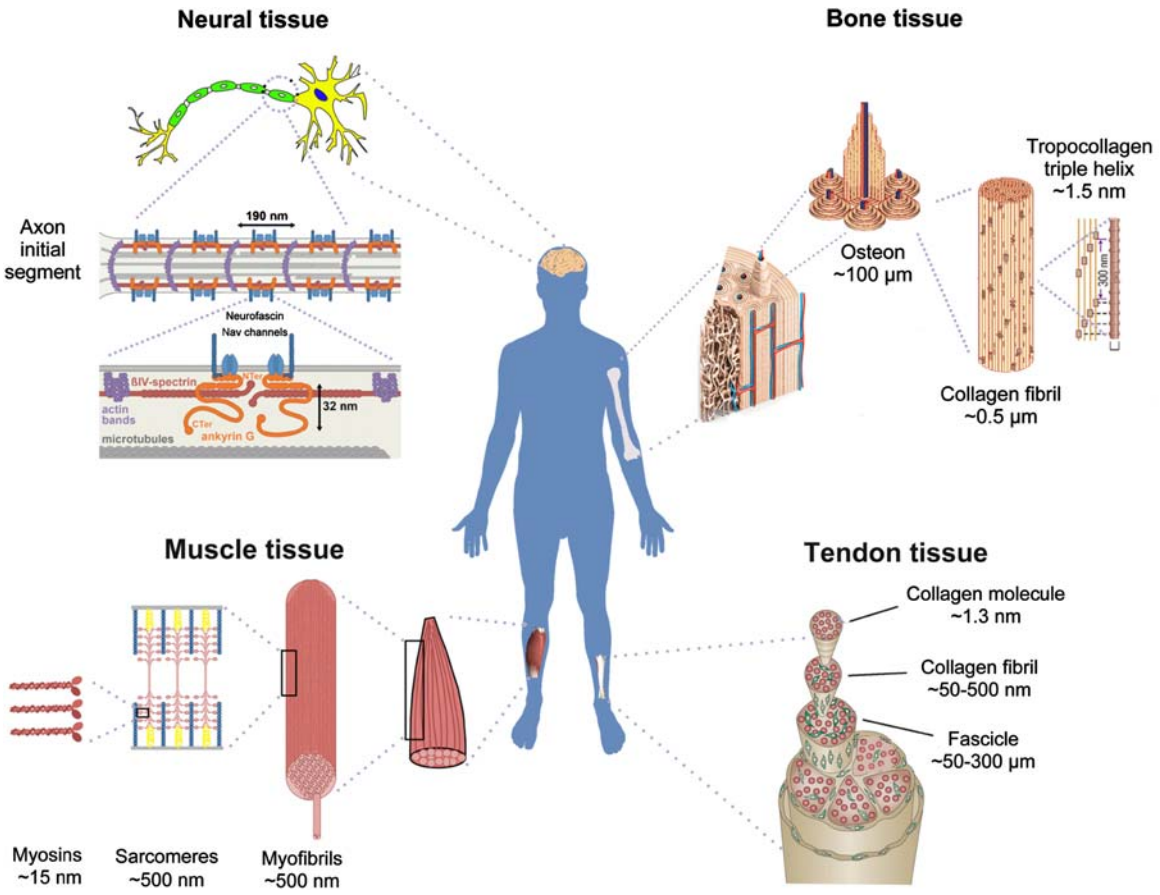

FIGURE 29.1 **Nanoscale features of human tissues.** In neural tissue, the axon initial segment is responsible for generating action potentials in neurons and is organized at the nanoscale. This structure is organized periodically in a longitudinal direction and is layered radially. This nanoscale architecture is crucial for neuronal function. (Adapted from Leterrier et al. [2].) The compact bone tissue consists of osteons (100 μm), which are composed of reinforced fibers formed by aggregated type I collagen and calcium phosphate nanoparticles (Adapted from Wegst et al. [3].) In muscle tissue, myofibrils are aligned in parallel and are composed of sarcomeres. The sarcomeres consist of protein filaments composed of actin and are arranged in a direction parallel to thick filaments composed of aggregates of myosin. Myofibrils, sarcomeres, and myosin can have nanoscale dimensions. In tendon tissue, a tendon fiber is composed of a hierarchical structure of tendon fibers, fascicle, and nanoscale collagen fibrils (50–500 nm), which are further composed of collagen molecules (~1.3 nm). *Adapted from Nourissat G, Berenbaum F, Duprez D. Tendon injury: from biology to tendon repair. Nat Rev Rheumatol 2015;11:223–33.*

PROPERTIES OF NANOMATERIALS

One aim of regenerative medicine is to embed cells in synthetic or natural scaffolds to produce functional tissues or organs. In the body, cells interact closely with the nanostructured extracellular matrix (ECM), where these nanoscale topographical features can direct cellular growth, adhesion, motility, and differentiation [5]. Cells also adhere to proteins located in the nanofibrous ECM, such as laminin and fibronectin, by their nanoscale receptors such as integrins and cadherins [11]. To fabricate biomimetic scaffolds that can be used to improve tissue regeneration, nanomaterials such as nanoparticles, nanofibers, and nanofilms have been developed to mimic the composition and structure of tissues at the nanoscale [4,5]. These nanomaterials possess distinguished physicochemical, optical, magnetic, and electrical properties compared with their bulk-level counterparts [12] (Fig. 29.3). Next, we will discuss these unique properties of nanomaterials that have enabled novel applications to develop functional tissues, targeted delivery, and in vivo stem cell imaging for tissue regeneration.

Physical Properties

The physical properties of natural systems, such as their size, shape, and surface topography, are directly related to their function. For example, the discoidal shape of erythrocytes promotes its mechanical flexibility and

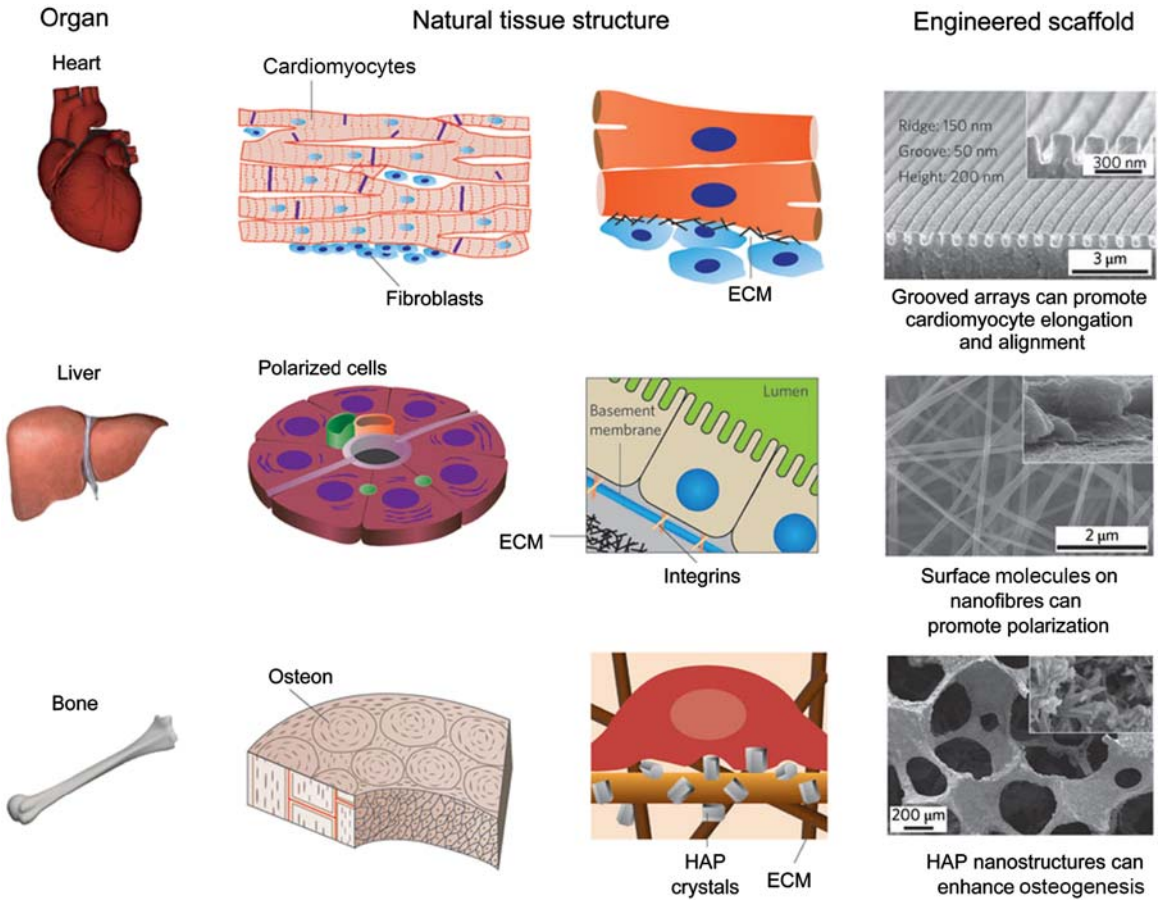

FIGURE 29.2 **Engineering nanostructured scaffolds for human tissues.** Heart tissue is composed of elongated and aligned cardiomyocytes that are mechanically coupled with the nanostructured extracellular matrix (ECM) to form anisotropic syncytium. Nanogrooved structures can be formed on substrates to promote the alignment of cardiomyocytes for cardiac tissue engineering. Liver tissue is composed of polarized epithelial cells between the ECM, lumen, and other cells for efficient mass transfer. This tissue polarity can be recapitulated on nanofibers by surface modification to enhance cell adhesion. Bone tissue is a composite of collagen and hydroxyapatite (HAP) nanocrystals. A bone-mimetic scaffold composed of HAP can promote osteogenesis and bone formation. *Adapted from Dvir T, Timko BP, Kohane DS, Langer R. Nanotechnological strategies for engineering complex tissues. Nat Nanotechnol 2011;6:13–22.*

biorheological properties and allows them to traverse blood vessels as small as 2–3 μm [13,14]. In addition, the surface texture of cell substrates, particularly at the nanoscale, can influence various cellular processes such as differentiation, motility, spreading, and apoptosis [13]. For example, epithelial cells grow in an elongated form on surfaces patterned with nanoscale grooves and ridges, whereas these cells have a rounded morphology on smooth surfaces [11]. These observations have promoted the development of nanomaterials with novel physical properties for applications in regenerative medicine.

Size

The delivery of drugs, genes, and peptides to a specific tissue can enhance drug efficiency while lowering systemic side effects; it is a useful approach in regenerative medicine for treating diseased or damaged tissues [5]. The small size of nanoparticles results in favorable properties compared with microparticle-based delivery methods, such as improved circulation and the ability to cross tissue barriers [15]. The size of the nanoparticle is correlated with the efficiency of cellular uptake and is determined by the avidity of ligand–receptor interactions between the nanoparticle and the cell surface [16]. In the range of 5 nm, nanoparticles cannot bind a sufficient number of receptors to induce membrane wrapping for efficient uptake. However, nanoparticles of sizes 50 nm or greater can bind multiple receptors but can limit the additional binding of nanoparticles. These properties can vary according to the cell type and culture conditions, which can cause variations in the number of receptors and internalization mechanisms [16].

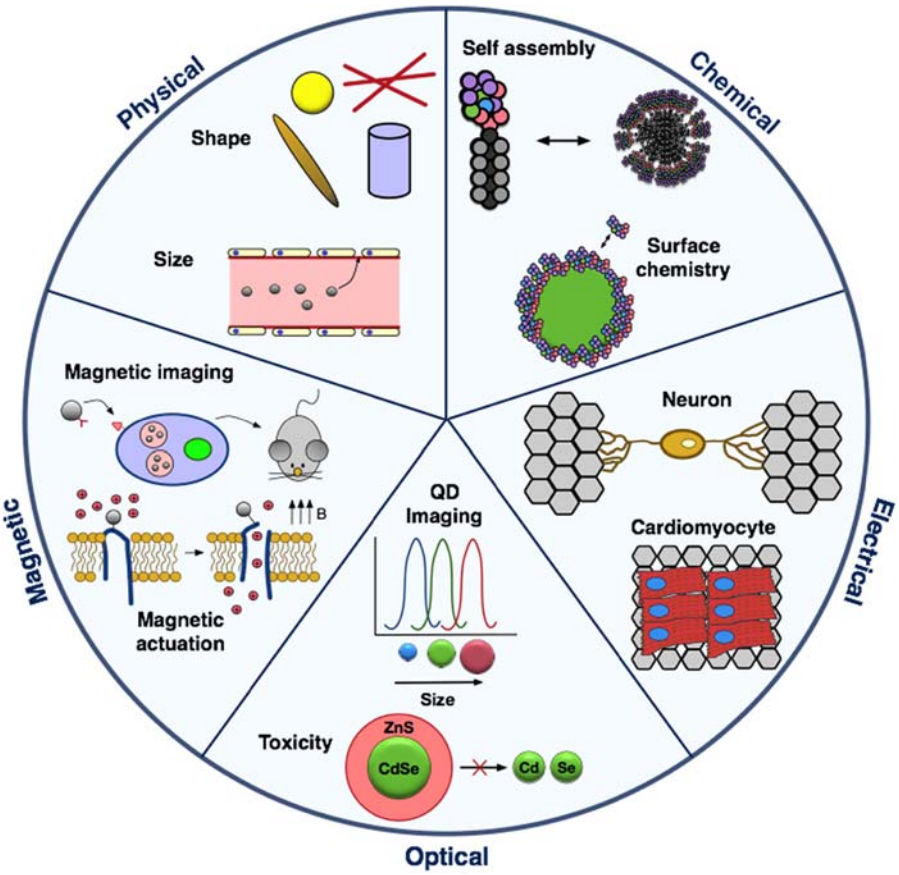

FIGURE 29.3 **Physicochemical properties of nanomaterials.** The unique physical features of nanomaterials include their size and shape. The small size of nanoparticles allows them to traverse tissue barriers. The shape of nanoparticles can vary from particles to fibers, needles, and tubes. These nanomaterials can self-assemble into a larger structural unit based on covalent or noncovalent interaction. For example, peptides with an amphiphilic structure can self-assemble into tubular structures. The high surface area and reactivity of nanoparticles can promote protein adsorption, which can affect their biological properties. Electrically conductive nanomaterials can be applied to restore the electroactive function of tissues such as nerve and heart. The optical properties of quantum dots (QDs) create variations in color according to size properties. The toxicity of QDs can be minimized by coating with a bioinert ZnS layer. The magnetic nanoparticles can be used for magnetic resonance imaging and can control magnetic actuation of individual receptors with a magnetic field (*B*).

The regeneration of brain tissue is particularly limited by the extremely low permeation rate (<1%) of small drugs or peptides, such as neurotrophins, through the blood–brain barrier (BBB) [17]. This barrier is formed by tight junctions between blood vessel endothelial cells (ECs), which restrict the passage of solutes [17]. To pass the BBB, gold nanoparticles (AuNPs) can be a potentially powerful drug carrier owing to their small size. In particular, 15-and 50-nm AuNPs could accumulate in the brain [18], which demonstrates their possible use in crossing the BBB for drug delivery applications.

Shape

The anisotropy and shape specificity of biological interactions are a critical parameter for functions at the cell, tissue, and organ levels [19]. For example, hydroxyapatite (HAP) ($Ca_{10}[PO_4]_6[OH]_2$) nanoparticles with a needle shape incorporated in a polycaprolactone (PCL) scaffold can promote osteoblast differentiation compared with rod or spherical-shaped HAP owing to their improved mechanical strength [20,21]. Therefore, the modification of nanoparticles into specific shapes such as cylinders, rods, and tubes has resulted in novel applications for tissue engineering.

In some cases, the shape of a nanoparticle can have a more significant effect than size on its functionality. For example, although micrometer-sized particles are cleared rapidly from circulation, 18-μm-long filomicelles with two nanoscale dimensions (20–60 nm) have a dramatically improved circulation half-life (∼5 days). This unique property allows filomicelles to cross tissue barriers such as in the spleen and to evade phagocytosis and degradation by the mononuclear phagocyte system (MPS) [22].

The incorporation of anisotropic nanostructures using materials such as carbon nanotubes (CNTs) and nano-HAP into scaffolds can also mimic the anisotropic structure of the ECM to provide ex vivo cues for tissue development [19]. For example, collagen fibrils in the ECM can be hundreds of micrometers long with diameters on the nanoscale (10−300 nm) and their longitudinal orientation results in high tensile strength. Similarly, CNTs have a high aspect ratio and their aligned structure in polymer scaffolds can impart high mechanical strength to scaffolds [23,24].

Surface Topography

Interest in adult and embryonic stem cells (ESCs) for applications in regenerative medicine has grown considerably, particularly for their ability to differentiate into multiple cell types to produce new tissues [25]. Materials with nanoscale topography can control cell behavior [25], based on the similar mechanism that cells can detect physical and topographical cues at the nanoscale of the ECM, such as cross-linking patterns, fiber length, fiber diameter, and surface irregularities [26]. This interaction occurs at the level of individual receptors on the cell surface, such as the transmembrane, heterodimeric protein receptors consisting of α- and β-integrin subunits [27]. These physical cues can guide cell orientation, adhesion, and differentiation, which are essential for the formation of functional tissues [5].

In a process termed "nanoimprinting," the shape and topography of the substrate (e.g., grooves, ridges, pits) can induce a similar shape in cells through integrin clustering and cytoskeletal reorganization [27,28]. For example, a nanoimprinted surface of grooves and ridges could promote the neurite growth and alignment of dorsal root ganglion neurons, which is a useful approach to sensory neuron regeneration [29]. The scale length of the substrate is also correlated with cell activity, because nanoscale island shapes ranging from 10 to 20 nm can promote cell adhesion and spreading of fibroblasts, mesenchymal stem cells (MSCs), and ECs, whereas a size of 100 nm can inhibit cell spreading and differentiation [27]. In accordance with this general observation, a spacing of 15 nm could promote the osteogenesis of MSCs owing to optimal integrin clustering [30].

Cell−substrate interactions can induce mechanical tension (i.e., mechanotransduction) and nuclear reorganization through cytoskeletal tension, resulting in gene expression changes that influence stem cell differentiation and function [31]. The influence of mechanotransduction on gene expression is a result of multiple synergistic effects such as the increased access of transcription factors to DNA, the alteration of nuclear pores for messenger RNA transport, and transcription complex formation [32]. A number of pathologies result from the loss of mechanotransduction, such as osteoporosis, cancer, and arteriosclerosis, which demonstrates its importance in cellular function [33].

In addition to physical signals, the interaction of cells on nanostructured substrates can induce chemical signals, particularly through the integrin-linked signal cascade [32]. The binding of integrin receptors to motifs in the ECM (e.g., the arginine-glycine-asparate [RGD] motif in fibronectin, vitronectin, and laminin) results in their clustering into supramolecular complexes, called focal adhesions [34]. The focal adhesion kinase (FAK) protein, which is a nonreceptor tyrosine kinase, remains constitutively bound at these sites through interaction with the β-integrin subunit. As an adhesion-dependent kinase, FAK can phosphorylate a number of molecules, such as the extracellular signal-regulated kinase (ERK) 1 and 2, to affect downstream cell signaling [32]. For instance, ERK 1/2 can translocate into the nucleus and modulate cellular activity by affecting transcription factor expression, such as the runt-related transcription factor 2, a key regulator of bone formation [32,35]. Evidence that the nanotopographical interaction of substrates can influence cell activity has promoted the development of novel nanostructured biomaterials for applications in bone and heart tissue regeneration.

Bone tissue is a nanocomposite structure of organic collagen nanofibers and inorganic materials such as HAP and whitlockite ($Ca_{18}Mg_2[HPO_4]_2[PO_4]_{12}$), which range in size from 20 to 50 nm in lamellar bone and 10 to 50 nm in woven bone [36−38]. However, conventional orthopedic bone implants are based on larger, micrometer-scaled grains, which can cause fibrotic tissue formation as a result of poor bone tissue integration [36]. The formation of a nanostructured coating layer on the surface of bone implant materials (i.e., ceramics and metals) can improve their biocompatibility and bioactivity by stimulating osteoblast adhesion, proliferation, and calcium deposition [5,36]. In particular, surfaces with random features at the scale of 100 nm can promote greater osteogenesis of human MSCs (hMSCs) compared with ordered surface, and can be incorporated into biomaterials for improved bone−material contact [27,39,40].

The myocardium of the heart is composed of many different cell types, such as cardiomyocytes and fibroblasts, which interact with the nanoscale topographical and molecular structure of the surrounding ECM [11]. In particular, the nanoscale structural and mechanical cues of the ECM can promote mechanical coupling between cardiomyocytes to form aligned, elongated bundles to create an anisotropic syncytium [11]. However, the in vitro expansion of cardiomyocytes results in the loss of this native organization and can compromise features crucial to tissue

function, such as cell geometry and conduction velocity [41]. The formation of cardiac tissue constructs with nanoscaled grooves can recapitulate cardiomyocyte elongation and alignment found in vivo through increased contraction-mediated stress and cell spreading [41].

Chemical Properties

The surface atoms of bulk solids represent a small fraction of their total composition, so the external surface contributes minimally to their material properties. However, as the surface-to-volume ratio increases to the nanoscale, the surface chemistry becomes dominant, affecting chemical properties such as solubility, luminescence, and catalytic activity [42].

Surface Chemistry

As a result of their surface reactivity, nanoparticles can adsorb endogenous proteins on their surface when placed in biological environments [43]. In the blood circulation, nanoparticles adsorb serum proteins such as immunoglobulins, complement factors, and lipoproteins, which can act as an opsonin for recognition by the immune system ultimately to influence its biodistribution [44]. In particular, negatively charged nanoparticles have a reduced level of serum protein adsorption, which can increase their circulation half-life and reduce accumulation in the liver and spleen [45,46]. In contrast, positively charged nanoparticles rapidly adsorb serum proteins, which act as a signal for clearance by the MPS in the spleen and liver [16]. For this reason, the grafting of hydrophilic polymers such as poly(ethylene glycol) (PEG) is a promising strategy for improving circulation time by blocking protein adsorption through steric hindrance [47].

The internalization of nanoparticles into cells also depends on its surface charge. In particular, positively charged nanoparticles demonstrate efficient cell uptake ability compared with neutral or negative nanoparticles because of favorable electrostatic interactions with the negatively charged cell surface [16]. The interaction with positively charged particles can induce local fluidity of the phospholipid bilayer, indicating a potential mechanism for the transportation of absorbates across the cell membrane [48].

Self-assembly

Nanostructures can be formed through the self-assembly of intricate and ordered structures through weak noncovalent forces such as ionic bonds, hydrogen bonds, hydrophobic interactions, and van der Waals interactions [49]. This bottom-up approach to the formation of supramolecular architectures such as collagen, keratin, and coral is common in nature and can be harnessed to fabricate novel biomaterials [49]. For example, amphiphilic peptides can form β-sheet structures through complementary interactions between their hydrophobic and hydrophilic surfaces. These nanoscale fibers (10−20 nm in diameter) result in an interwoven matrix and can function as a cell scaffold for tissue regeneration [49,50]. In particular, these scaffolds can mimic the gross architecture of the ECM, with a small pore size (5−200 nm) and high water content, which allows growth factors and nutrients to diffuse slowly and cells to reside in a three-dimensional (3D) environment [49].

Optical Properties

The transplantation of stem cells is an emerging therapeutic strategy in regenerative medicine to guide tissue regeneration [12]. An important factor in this approach is the development of noninvasive, biocompatible, and long-term strategies for monitoring stem cell proliferation, differentiation, and incorporation into the target tissue [12]. The development of nanomaterials such as quantum dots (QD) has enabled cell-specific imaging techniques with high spatial resolution and sensitivity for stem cell−based therapies [12].

QDs are colloidal semiconductor nanocrystals composed of compounds such as cadmium sulfide, cadmium selenide (CdSe), and indium phosphide [51]. At the scale of QDs (a few nanometers), quantum effects become more prominent, producing advantages over traditional fluorophores, such as an increased fluorescence life span and high fluorescence intensity [12,51]. These unique advantages can enable the long-term labeling of stem cells after they are transplanted or injected into the desired location. For example, QDs functionalized with RGD peptides remained labeled in hMSCs during self-replication and differentiation along adipogenic, androgenic, and osteogenic lineages [52]. In addition, QDs can enable the tracking of stem cells during their mechanical integration with surrounding tissue. The delivery of QD-labeled hMSCs to the heart demonstrated clear spatial distribution without nonspecific targeting to neighboring cells and could be imaged after 8 weeks [53]. In addition, the emission

properties of QDs could be altered based on their size and composition, from blue wavelengths to the near-infrared region [54]. This unique property of QDs allows for multiplex imaging of various cell types in a single organism using multiple types of QDs. For example, ESCs can be labeled with multiple QDs ranging in emission from 525 to 800 nm and can be simultaneously imaged using a single excitation wavelength [55].

However, a high concentration of QDs can lead to toxicity issues such as malformations in embryonic development [55a]. The release of heavy metal ions such as Cd^{2+} or Se^{2-} from CdSe-based QDs or the generation of free radicals by photoirradiation can have a detrimental effect on cell viability [51,56]. Therefore, techniques to reduce the toxic effects of QDs, such as coating with bioinert ZnS to block the release of Cd^{2+} ions, are essential for future clinical applications [56,57].

Magnetic Properties

Magnetic nanoparticles (MNPs) are a class of nanomaterials composed of metals such as cobalt, nickel, and iron, with paramagnetic, ferromagnetic, or superparamagnetic properties [58]. In particular, superparamagnetic iron oxide nanoparticles (SPIONs) are promising for in vivo applications because they are composed of biocompatible iron metals and do not agglomerate because of a lack of mutual attraction [58]. These nanoparticles have multifunctional applications in regenerative medicine, such as stem cell tracking and nanomagnetic actuation of cell surface receptors.

The high sensitivity of SPIONs as a contrasting agent in magnetic resonance imaging (MRI) can enable more effective in vivo stem cell tracking [59]. The modification of SPIONs with transfection agents such as poly-L-lysine can promote efficient internalization into cells, reducing the need for high concentrations that can induce cytotoxicity [60]. The transfer of magnetic properties to cells may enable the systematic study of delivery methods, optimal engraftment approaches, and cell migration for applications in stem cell therapy [58].

In addition, MNPs can attach to the cell membrane and activate mechanosensitive ion channels when a strong magnetic field is applied, which can be used to control cellular processes [61]. In particular, physical forces such as pressure-induced strain can direct the differentiation of MSCs into various lineages such as bone, muscle, and connective tissue [61,62]. The advantage of MNPs in this approach is their small size, which can allow the mechanical actuation of individual cell receptors by an external magnetic field [61]. For example, MNPs can be targeted to the mechanosensitive potassium ion channel TREK with anti-TREK antibodies and could promote the osteogenesis of MSCs when applied with a cyclic (1-Hz) magnetic field [63].

Electrical Properties

The incorporation of nanomaterials such as CNTs and graphene can increase the conductivity of scaffolds to promote the regeneration of electrically active tissues [11]. For example, CNTs are a promising material for use in neural implants because they are highly conductive and can promote the transmission of electrical signals between neurons [5]. In particular, CNTs can interface closely with neuronal circuits and synapses and can promote the formation of functional synapses [64]. Graphene has also been used to produce 3D conductive scaffolds for neuroregeneration [65]. In a model of the mouse hippocampus, graphene-based scaffolds could promote the growth of neurons and enhance their branching and outgrowth [65].

Because heart muscle tissue is underlined with conductive Purkinje fibers, the incorporation of conductive nanomaterials is relevant for myocardial tissue engineering [66,67]. For example, the homogeneous incorporation of CNTs in gelatin-based scaffolds can improve cardiomyocyte adhesion, maturation, and alignment [67]. Furthermore, to provide conductivity in cardiac scaffolds for the improved electrical communication of cardiomyocytes, they can be coupled with conductive materials such as gold nanowires [68].

NANOBIOMATERIALS

Nanobiomaterials describe biomaterials with a size between 1 and 100 nm in at least one dimension [69]. A major advantage of nanobiomaterials is their high surface-to-volume ratio, which is important for wound healing applications because a minimal amount of material can be used to cover a large area of defect while providing an extensive surface area to deliver drugs [70,71]. Nanostructured biomaterials can also interact directly with nanoscale cell surface receptors and cellular components to provide instructive signals to the cell. Nanobiomaterials have also

been applied in regenerative medicine because they can recreate the composition and structure of the ECM at the nanoscale. Nanobiomaterials can also improve the properties of engineered tissue constructs, such as mechanical strength [11,72]. For example, nanoclay and nano-HAP have been widely used as reinforcing fillers to strengthen the mechanical stability of dental implants and articular grafts [73]. Therefore, nanobiomaterials have great potential in regenerative medicine based on their ability to direct cellular activity [74].

Nanobiomaterials can be classified as metallic, inorganic-based, carbon-based, polymeric-based, and biological protein−peptide-based, depending on their compositions (Table 29.1) [75].

Examples of metallic nanobiomaterials include AuNPs, silver nanoparticles, and iron oxide nanoparticles [75,76]. AuNPs are useful for their simple synthesis techniques, adjustable optical properties and good biocompatibility [77]. In addition, AuNPs can convert absorbed light into heat via a series of nonradiative electron relaxation dynamics [78,79]. For these reasons, they have been broadly applied in various applications, particularly drug delivery [80], photothermal cancer therapy [81], and cell tracking [82]. Advantages of silver nanoparticles include their antimicrobial and antiinflammatory properties, which are useful for combating infections; these properties are further enhanced by their large surface area [83]. Silver nanoparticles can also accelerate wound healing owing to their antimicrobial activity and can be used in artificial joint replacements by reducing the levels of proinflammatory cytokines and minimizing the wear of implants [84]. In addition, iron oxide nanoparticles are a type of magnetic nanoparticle that can be employed to transfect DNA and antisense oligonucleotides for gene therapy [85,86].

Inorganic based nanobiomaterials include mesoporous silica nanoparticles and HAP-based nanobiomaterials [75,76]. Mesoporous silica nanobiomaterials are widely used in regenerative medicine applications such as drug delivery [87] and gene delivery [88] owing to their good biocompatibility and highly porous framework. In addition, mesoporous silica nanobiomaterials can be manufactured into different shapes, such as rod-like particles, fibers,

TABLE 29.1 Examples and Applications of Nanobiomaterials

Classifications	Examples	Applications	References
Metallic nanobiomaterials	Gold nanoparticles; silver nanoparticles; iron oxide nanoparticles	Drug delivery; photothermal therapy (breast cancer, skin carcinoma, and leukemia); detection of inflammatory and degenerative disease; cellular labeling and separation	[80−82,104−108,85]
Inorganic-based nanobiomaterials	Mesoporous silica nanoparticles; hydroxyapatite-based nanobiomaterials; carbon-based nanobiomaterials	Drug delivery; gene delivery; intracellular pathogen treatment; dental restoratives; bone defect repair platforms	[88,93,109−113]
Carbon-based nanobiomaterials	Carbon nanotubes; graphene; nanodiamonds; buckminster fullerene	Drug delivery; photothermal ablation of tumors	[76,113a]
Polymeric-based nanobiomaterials	Natural polymer-based nanobiomaterials (sodium alginate, starch, hyaluronic acid, chitin/chitosan and derivatives, dextran, albumin, and gelatin); synthetic polymer-based nanobiomaterials (poly[D,L-lactic acid], poly[D,L-lactic-*co*-glycolic acid]), poly (hydroxylalkanoates), polycaprolactone, polyacrylamide, polyanhydride)	Drug delivery; gene therapy; sensing; tissue regeneration	[98−100,114,115]
Biological protein −peptide-based nanobiomaterials	Gelatin; albumin; elastin; milk protein; gliadin; legumin	Gene delivery; gene therapy; antimicrobial substances to treat brain infections; antimicrobial substances to treat infectious diseases; intracellular delivery of proteins	[101,103,116−119]

films, and hollow and solid nanospheres for various functionalities. For example, nanotubes can be differentially functionalized based on their outer and inner surface [89]. Furthermore, because HAP is the most common inorganic biomineral in bone tissue [90], HAP-based nanobiomaterials are a promising candidate for dental restoratives and bone defect repair platforms [91,92]. In general, HAP-based systems can be combined with collagen or elastin nanofibers to recreate the composition and structure of bone tissues to treat bone defect sites. These systems are often loaded together with antibacterial drugs or growth factors, particularly bone morphogenic protein-2, to further enhance bone tissue regeneration [93].

Carbon-based nanobiomaterials mainly refer to CNTs, graphene, nanodiamonds, and buckminster fullerene (C_{60}), which possess strong optical absorption, good electrical conductivity, and high mechanical strength [94,95]. In addition, the surfaces of these carbon-based nanobiomaterials can be functionalized by oxidation treatments or conjugation with bioactive molecules to improve their solubility and biocompatibility [96].

Polymer-based nanobiomaterials can be fabricated into different structures and compositions; they possess a range of properties that are suitable for wide applications in regenerative medicine, such as bone plates, drug delivery carriers, and tissue engineering scaffolds [97]. For example, the surfaces of polymer-based nanobiomaterials can be modified with ligands of bovine serum albumin, glutathione, transferrin, or alkane thiols to develop actively targeted drug or gene delivery systems for enhanced therapeutic efficiency in biomedical applications [98–100].

Polymeric nanobiomaterials can be further categorized into natural biological and synthetic polymer-based nanobiomaterials. Compared with synthetic polymer-based nanobiomaterials, natural polymer-based nanobiomaterials have better biocompatibility, which is beneficial for cell adhesion and tissue regeneration. However, natural polymer-based nanobiomaterials can exhibit poor mechanical properties, such as low strength and stiffness, which can result in the instability of polymer-based nanobiomaterials and burst drug release within biological systems [76]. In comparison, synthetic polymer-based nanobiomaterials have greater mechanical strength and improved structural integrity, but they generally have lower biocompatibility compared with natural polymer-based nanobiomaterials [75]. Therefore, the concept of blended polymeric nanobiomaterials composed of natural and synthetic polymer-based nanobiomaterials was devised to meet both of these biological and physical requirements [97].

Biological protein–peptide-based nanobiomaterials mainly include gelatin, albumin, elastin, milk protein, gliadin, and legumin [76]. Biological protein–peptide-based nanobiomaterials can be applied as nanocarrier systems because they are biodegradable, biocompatible, stable in blood circulation, and amphiphilic for favorable interactions with drugs and solvents [101]. In addition, biological protein–peptide-based nanobiomaterials have the flexibility to acquire desirable biophysical characteristics by surface modifications such as self-assembly or binding with other various nanomaterials [102]. For example, antimicrobial cationic peptides can be self-assembled into core-shell nanoparticles and have enhanced antimicrobial properties to treat infectious diseases with a high therapeutic index [103].

NANOTECHNOLOGY-BASED STRATEGIES IN REGENERATIVE MEDICINE

Bone Tissue

Bone tissue forms the human skeletal system of a vertebrate, supporting the whole body, protecting organs, producing blood cells, and storing minerals. Bone tissue can be maintained in a healthy state based on continuous bone remodeling processes [120]. Specifically, osteoclasts can dissolve weak and old bone regions by locally secreting acidic substances such as protons, whereas osteoblasts can form new bone tissues by secreting proteins such as collagen. However, disruption of these cell activities, as well as various bone diseases such as nonunion bone fractures, osteoarthritis, osteoporosis, or bone cancers, can cause pain and disability.

Bone minerals incorporated in implants are commonly used to treat bone defect sites [120a]. For instance, synthetic HAP is a widely used component of bone implants because it is similar in composition to natural bone minerals [120a]. In an organic–inorganic hybrid, HAP incorporated in a poly(lactic acid) (PLA) scaffold can be fabricated by homogeneously mixing these two components in an ether-based solution, using sonication and freeze-drying to maintain its nanostructure [72]. These polymeric constructs have a high porosity (>90%) that can enhance cell ingrowth and survival, and the incorporation of HAP nanocrystals can improve the mechanical properties of the hybrid scaffold [72]. Furthermore, these HAP nanocrystals increase the adsorption of proteins, such as fibrinogen or bovine serum albumin, which can promote the osteogenic differentiation of MSCs [6].

The surface nanotopography of bone implants can improve osseointegration, which is the formation of a direct interface between implant materials and bone. For example, by using colloidal lithography techniques, titanium rods can be patterned with 60-nm polystyrene semispherical protrusions to enhance bone–implant contact [121]. When this nanostructured implant was placed in a rat tibial bone defect model, it showed higher bone–implant contact compared with the nonpatterned control group. Interestingly, when the size of protrusions was increased to 120 nm, bone–implant contact decreased, which suggested an optimal size range for bone cell contact. In addition, the formation of a coating layer with osteogenic nanoparticles can further enhance bone regeneration. For example, implant materials such as polyether ether ketone and titanium have mechanical properties similar to that of bone and have been applied to treat bone in the clinic [122,123]. However, these materials are biologically inert and can separate from the surrounding tissues after implantation. In this respect, the formation of a coating layer with bioactive nanomaterials such as HAP nanoparticles can enhance the attachment and growth of osteoblasts, promoting osseointegration and bone regeneration [124].

Nanoparticle-based drug delivery can be also used to treat osteoporosis and bone cancer. AuNPs are a widely used drug delivery vector because of their low toxicity and capability for surface functionalization [80,77]. In addition, AuNPs are known to accelerate the osteogenic differentiation of stem cells while suppressing the formation of osteoclasts [125,126]. AuNPs can also be readily functionalized with biological molecules by surface modification [127]. For example, AuNPs can inhibit the activity of osteoclasts and load and deliver alendronate (ALD), an antiresorptive drug that can induce new bone formation by promoting the proliferation and differentiation of osteoblasts [128]. ALD-conjugated AuNPs can easily be obtained by mixing AuNPs with ALD solution [129]. These ALD-conjugated AuNPs can enter cells by endocytosis and can enhance the formation of trabecular bone when applied in osteoporotic mice. In addition, dextran-based lipid nanoparticles can be used to deliver anticancer drugs such as DOX [130]. This DOX-loaded polymeric nanocarrier has a curative effect on drug-resistant osteosarcoma cell lines through the accumulation of drugs in the cell nucleus. As a result, DOX-loaded nanoparticles can increase the apoptosis rate of osteosarcoma cells compared with applying DOX alone.

Muscle Tissue

According to its function and location in the body, muscle tissue can be divided into three types: skeletal, smooth, and cardiac muscle tissues [131]. Skeletal muscle is responsible for carrying out movements and maintaining the posture of the body [132]. Smooth muscle can support hollow organ contractility, such as the gastrointestinal tract, the bladder, and blood vessels [133]. Finally, cardiac muscle is the muscle of the heart, which is autonomically regulated to self-contract in rhythmic fashion for the whole life of the organism [134].

Diseases related to dysfunction of muscle tissue include age-related muscle dysfunction, muscular dystrophy, localized muscle fatigue, muscle dysmorphia, muscle fiber necrosis, and progressive muscle weakness [135–139]. In addition, common clinical situations such as aggressive tumor ablation and severe denervation can result in significant muscle tissue loss, which can require additional surgical reconstruction. However, only few treatments can restore damaged functional muscle tissues effectively [140]. Among these treatments, free tissue transfer is widely used in the clinic, which is the transfer of living tissue obtained from a specific region of the body (e.g., skin, bone, tendon, muscle, and fascia) to another damaged region of the body through anastomosis to facilitate the reconstruction of complex defects [141]. However, limitations exist with free tissue transfer, particularly morbidity of the donor site [140]. In this respect, nanotechnology-based engineered tissue can recreate nanoscale features of the ECM to replace damaged muscle tissues [142,143].

To regenerate muscle tissue, various nanotechnologies have been employed to assemble nanobiomaterials, such as electrospinning [144], polyelectrolyte thin film deposition [145,146], and cell sheet technology [147]. Electrospinning is a fabrication technique that can be used to create complex 2D and 3D structures, based on ultrafine nanofibers [148]. Various types of polymers including collagen, cellulose acetate, PLA, poly(lactide-*co*-glycolide) (PLGA), PCL, and polyvinyl alcohol can be electrospun with diameters ranging from smaller than 3 nm to over 1 μm [149]. The seeding of myoblasts on the surfaces of parallel nanofibers is analogous to the native histological organization of skeletal muscle tissue and is beneficial for generating aligned cell layers to enhance myogenic differentiation [150].

In thin-film deposition, gaseous, liquid, or sol-gel precursors of biomaterials are deposited onto a given substrate; the film thickness can be controlled to within few tens of nanometers [151,152]. For example, PEG-linked multiwalled CNT films with nanoscale surface roughness can be engineered using the thin-film deposition method. This nanostructured film can resemble the nanoarchitecture of the ECM, which can facilitate protein adsorption and stem cell attachment and promote the differentiation of hMSCs to the skeletal myogenic lineage [152].

In the case of cardiac muscle tissue, it allows for the continuous beating of the heart because of its high contractile strength and endurance and includes tissues such as the myocardium, endocardium, and pericardium [153]. The damage of cardiac muscle tissue can cause critical health issues such as stroke, aneurysm, and peripheral artery disease [154–156]. In addition, cardiac disease accounts for approximately 40% of global mortality [153]. Diseases and defects of the cardiac system include myocardial infarction, heart failure, cardiomyopathy, ischemic heart disease, congenital heart disease, hypertensive heart disease, and chronic heart disease [157,158].

Among these heart diseases, heart failure after a myocardial infarction (MI) is a major cause of death in the United States. In particular, approximately 3 million Americans experienced heart failure in 2015, and it is anticipated that 100,000 new cases will have been diagnosed in the following 5 years [159]. Total heart transplantation is a possible treatment for end-stage cardiac failure, but the lack of donor hearts is a major bottleneck for this approach. In addition, because damaged myocardial tissue caused by MI cannot undergo self-regeneration, the left ventricle cannot remodel successfully, resulting in heart failure [160]. The use of scaffolds that can repair the infracted tissues and regenerate new cardiac tissues based on patient cells can overcome the drawbacks of limited donor organs and immunological rejection [161].

Cell sheet technology is a nanotechnology that was developed to produce polymeric scaffolds for myocardial tissue engineering. This technique mainly depends on the use of poly(*N*-isopropylacrylamide) (PIPAAm), a biomaterial with a typical surface thickness less than 100 nm [147]. Because PIPAAm is a thermoresponsive polymer, when the environment is cooled to a temperature lower than the low critical solution temperature of PIPAAm (32°C), the material surfaces becomes hydrophilic and intact cells sheet can spontaneously detach and easily be harvested from the polymer substrate [162]. This scaffoldless cell sheet technology has been successfully applied for 3D myocardial tissue reconstruction by harvesting and layering cardiomyocytes, which suggests the advantages of nanoscale-thermoresponsive surfaces [147].

Vascular Tissue

Cardiovascular tissue has an important role in circulating blood for the transport of oxygen, carbon dioxide, nutrients, blood cells, and hormones, to maintain the homeostasis of human body. Because it is a vital organ of the human body to sustain life, disease and/or damage of vascular tissue can result in critical health issues. According to the Centers for Disease Control and Prevention (CDC), cardiovascular-related diseases resulted in over 596,000 deaths in 2011 which was the leading cause of death in the United States [163]. Therefore, artificial vascular grafts that can substitute for damaged blood vessels have been widely explored to treat these vascular diseases [164]. In particular, vascular grafts with nanostructured surfaces can promote cell adhesion and proliferation. For example, the surface roughness of PLGA vascular grafts can be controlled at the nanoscale through etching with NaOH solution. As a result, cell adhesion on this vascular graft is significantly enhanced, which can promote the proliferation of ECs and smooth muscle cells (SMCs) to improve tissue integration in vivo [165].

In addition, metal stents composed of titanium and nitinol are widely applied to treat vascular diseases such as atherosclerosis and superficial femoral artery stenosis, because of their durability and mechanical properties [166,167]. However, the adhesion of blood platelets on bare metal stents can result in in-stent restenosis [168]. To overcome this limitation, the surface of cardiovascular metal stents can be modified with nanopatterns to enhance the adhesion of human umbilical vein endothelial cells (HUVECs) for rapid reendothelialization in vivo. Because human blood vessels are formed from an inner layer of ECs as well as an outer layer of SMCs, nanopatterns that mimic the patterns of SMCs can be used to modify the surface of metal stents to promote endothelialization [168]. For instance, when fibril structures of nanoscale width (700 nm) were formed on stainless-steel stents using a femtosecond laser, the adhesion and proliferation of HUVECs were significantly enhanced. These surface-modified stents were almost fully covered by ECs within 30 days after implantation into an iliac artery–blocking model in a rabbit model.

Nanomaterial-based drug delivery can be also employed for the early detection and treatment of vascular diseases such as atherosclerosis and thrombosis, by targeting ECs or blood vessels [169]. In particular, dendrimers are highly branched globular macromolecules, and their multivalency enables the binding of multiple drug molecules to achieve a concentrated payload [170,171]. Dendrimers can be used for drug delivery by binding molecules on its surface or encapsulating drugs in their interior structure based on electrostatic or hydrophobic interactions [172]. For example, positively charged PEGylated dendrimers can electrostatically bind negatively charged heparins to treat deep vein thrombosis with increased circulation time [173]. When heparin was encapsulated in dendrimers,

the half-long period of heparin was more than doubled; this heparin−dendrimer complex could reduce 85% of thrombus in a rat jugular vein thrombosis model.

Neural Tissue

Neurons exist mainly in the brain and spinal cord and their nerves are distributed throughout the whole human body. Their function is to transmit and receive external and internal stimuli such as body temperature and light [174,175] and elicit responses in the form of action potentials [176]. The human nervous system is composed of two regions: the central nervous system (CNS) and the peripheral nervous system (PNS).

The CNS forms the brain and spinal cord and the PNS consists of nerves (autonomic, cranial, and spinal) that are connected to the CNS [177]. Unlike hard tissue such as bone, nerve tissue is a soft tissue and can easily be impaired. In fact, injury occurring in other tissues, such as bone fracture, are usually accompanied by nerve damage [178]. In addition, injuries of the CNS, such as cerebral ischemia, can result in neuronal death [179]. Similarly, neurological diseases such as Alzheimer disease (AD) can cause CNS disorders, resulting in memory impairment [180]. A main obstacle to treatment of the diseased or damaged CNS with drugs is the difficulty of passing through the BBB [181]. However, nanotechnology-based drug delivery strategy can be used to overcome this barrier to treat specific cells in the brain [182]. For example, AD, a chronic and progressive neurodegenerative disease, can be alleviated by rivastigmine. AD patients experience from memory loss, problems with language use, and emotion control; rivastigmine is known to improve or maintain their cognition and behavioral symptoms. To enhance the efficacy of this drug, poly(*n*-butylcyanoacrylate) (PBCA) nanoparticles with a polysorbate coating layer can be used as a nanocarrier [183]. PBCA is a biocompatible and biodegradable polymer that can entrap drugs to prevent rapid release [184]. Coating PBCA nanoparticles with polysorbate allows for efficient crossing of the BBB, because polysorbate can adsorb proteins such as apolipoprotein B/E, enabling uptake by the epithelial cells of brain capillaries by receptor-mediated endocytosis [185]. Based on this nanodelivery system, the concentration of rivastigmine in brain could increase more than three times after an intravenous injection in rats.

The PNS consists of nerves that connect the CNS, which extends to all regions of the human body. The injury of peripheral nerves interferes with communication between the CNS and damaged tissues, which can cause neuropathic pain and the functional loss of tissues. Nerve autografts are a substitute to repair injured peripheral nerve based on its low risk of immunological rejection and a rich amount of neurotrophic factors [186,187]. However, limitations of using autografts include the shortage of nerve autografts as well as donor site morbidity [188]. To overcome these limitations, scaffolds composed of aligned nanofibers can be used to treat PNS injury [189,190]. For example, aligned nanofibrous PLA scaffolds can be fabricated by electrospinning techniques. When neural stem cells (NSCs) are seeded onto this scaffold, their neurite outgrowth was parallel to the aligned PLA nanofibers. Interestingly, the neuronal differentiation rate of NSCs was higher for nanofibrous than for microfibrous scaffolds, which suggests that NSCs can preferentially interact with a nanostructured environment [189].

Other Tissue

Nanotechnology can be applied to other diseased or damaged tissues including cartilage, bladder, and skin. Because cartilage tissue lacks blood vessels and cells, it has a limited regenerative capacity [191], so external treatment is often required when damage occurs. Peptide amphiphiles (PAs) can be used to regenerate cartilage based on their capacity to bind a high concentration of cartilage growth factors. These PAs can self-assemble into supramolecular nanofibers with a high aspect ratio and can be designed to have a large number of binding epitopes for transforming growth factor-β (TGF-β), a chondrogenic growth factor. As a result, TGF-β−bound PA scaffolds can be used to direct the chondrogenic differentiation of MSCs to promote cartilage regeneration in vivo [192].

In addition, nanostructured scaffolds are applicable for bladder tissue regeneration. For example, nanoscale roughness can be generated on the surface of poly(ether urethane) and PLGA polymers by chemical etching to enhance the proliferation of urothelial cells and reduce bladder stone formation [193].

Skin is another important tissue because it acts as a barrier to protect interior tissues; serious health complications can occur when skin tissues are damaged by severe incisions or burn wounds. Because fibroblasts have an important role during the wound healing process by dissolving fibrin clots, producing collagen and ECM structures, and retracting wounds [194], nanofibrous scaffolds that support the growth of fibroblasts can be beneficial for skin regeneration [195]. For example, human dermal fibroblasts can proliferate on biodegradable nanofibrous scaffolds composed of poly(L-lactic acid)-*co*-poly(caprolactone) and gelatin and can improve collagen secretion.

NANOTECHNOLOGY-BASED STEM CELL THERAPY

Stem cell–based therapeutics can improve the treatment of human diseases [196]. The use of nanotechnologies to control stem cell fate accurately, improve in vivo delivery and integration, and promote expansion and proliferation has led to rapid advances in stem cell therapy in regenerative medicine (Fig. 29.4).

Stem Cell Transfection

Stem cells can be directed into multiple lineages to replace damaged tissues to restore their vital functions. Therefore, the ability to control stem cell fate in a precise manner has promoted therapeutic applications for regenerative medicine. This control is particularly crucial in clinical applications, because transplanted cells often have low viability and occasionally form malignant teratomas [197]. In particular, stem cell differentiation can be regulated by gene delivery using viral transfection [196]. Although viruses such as retroviruses, lentiviruses, and adenoviruses are highly efficient gene delivery vectors, they are limited because of high manufacturing costs and the potential for an immunogenic response [196].

The use of nanoparticles offers several advantages for the delivery of these factors compared with viral vectors, such as low immunogenicity, the ease of fabrication, and high gene loading efficiency [196]. Therefore, the in vivo delivery of genetic cues by nanoparticles can improve the therapeutic effect of stem cells by directing their differentiation into a specific cell type [196].

Stem cells are a useful approach to treating ischemic diseases because they can release angiogenic factors including vascular endothelial growth factor (VEGF) or participate directly in the formation of new blood vessels (i.e., vasculogenesis) in the case of endothelial precursors [198,199]. However, stem cell viability after transplantation is low, and their ability to form new blood vessels is limited owing to insufficient angiogenic gene expression [199]. To promote the angiogenic phenotype of hMSCs, the gene encoding VEGF can be delivered using biodegradable poly(β-amino ester) nanoparticles [199]. The transfection efficiency of this approach was superior to physical methods (e.g., electroporation) or other nonviral vectors (e.g., lipofectamine), and the transplantation of these

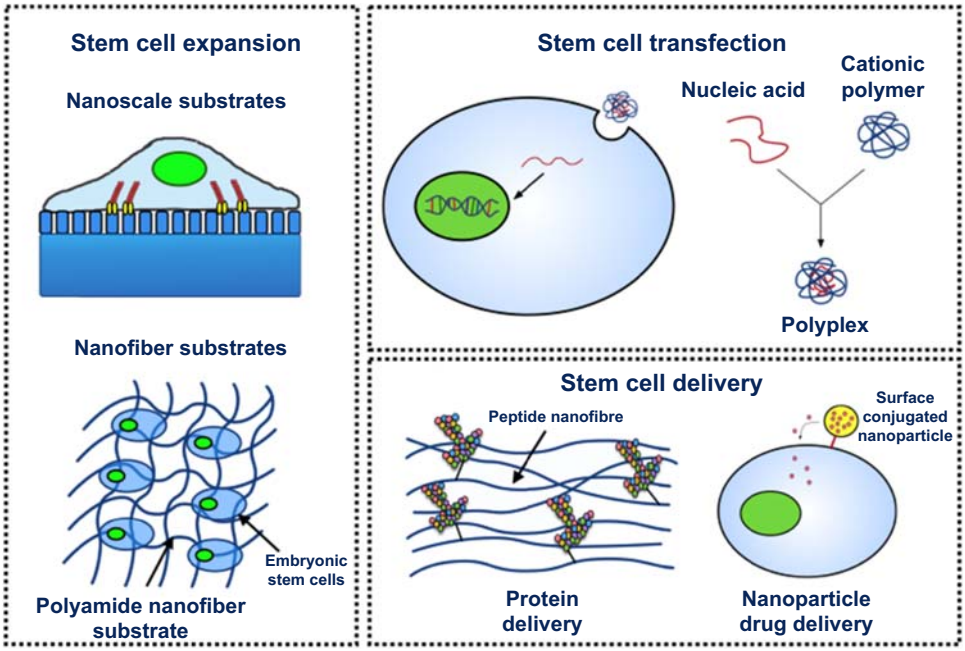

FIGURE 29.4 **Applications of nanotechnology for stem cell therapy.** Stem cell expansion in vitro can be promoted by nanoscale substrates, which can maintain their differentiation potential. Embryonic stem cells can be grown on polyamide nanofiber substrates to maintain their pluripotency and promote their proliferation. Stem cell transfection with nonviral polyplexes, which are nanoscale complexes of cationic polymers and anionic nucleotides, can enable genetic modification before implantation. Nanofiber substrates can also be used to enhance the recruitment of stem cells in situ by covalently attached peptides. Drug-loaded nanocarriers can be conjugated onto the cell surface and can improve stem cell delivery and promote their repopulation in vivo.

genetically modified stem cells could promote neovascularization in an ischemic mouse model [199]. These types of nanoparticles are termed polyplexes; they are formed by the interaction of a cationic polymer such as chitosan or polyethylenimine with negatively charged DNA to form structures with sizes of tens to hundreds of nanometers [200]. The close interaction of the polymer with DNA can promote its intracellular delivery and protection from degradation, resulting in transfection efficiency comparable to viral vectors [199,200].

Stem Cell Delivery

In addition to secreting trophic factors to promote tissue regeneration, transplanted stem cells can participate directly in the building of new tissues [201]. For example, after ischemic injury of the mammalian heart, MSCs can migrate to the myocardium to initiate repair by differentiating into cardiac muscle [202]. The integration of stem cells with host tissue requires multiple factors such as efficient migration from the site of injection, high cell viability, and effective engraftment into the target tissue [201]. However, the delivery of stem cells by injection, either systematically or at the tissue site, can result in high cell death and limited migration, resulting in low engraftment efficiency [201].

The incorporation of stem cells in a scaffold can improve the efficiency of transplantation by acting as a template to guide tissue regeneration, which can degrade simultaneously with the formation of new ECM by proliferating cells [201]. In particular, scaffolds based on nanofibers have a large surface area and high porosity and can affect cell behavior through interaction with nanoscale topographical features. These scaffolds can be constructed by electrospinning synthetic polymers such as PCL and PLA, or natural polymers such as chitosan and collagen, using an electric field on a rotating plate substrate [196]. In addition, nanofiber-based scaffolds can present bioactive factors to promote in situ tissue regeneration from the body's own stem cell and progenitor cell population [203]. In particular, to increase the adult stem cell population at the target tissue to accelerate tissue regeneration, they can be mobilized to the target site by cytokines and chemoattractants [203]. For example, stromal stem cell−derived factor-1α can be covalently bound to nanofibers composed of self-assembling peptides and can recruit endothelial progenitor cells for improved cardiac function by increased neovascularization [203,204].

The delivery of therapeutic stem cells often requires the concurrent delivery of adjuvant drugs such as TGF-β signaling inhibitors and γ_c receptor cytokines, which can promote their differentiation into an optimal therapeutic phenotype and enhance immune reconstitution [205]. However, the high dosage and systemic delivery required for these factors can cause toxicity issues because of their pleiotropic effects [205]. A clinically applicable alternative is to conjugate drug-loaded nanoparticles onto the cell surface, which can deliver low drug doses locally and follow the cell's migration patterns. For example, nanoparticles conjugated onto hematopoietic stem cells (HSCs) can locally release a glycogen synthase kinase-3β inhibitor to enhance the reconstitution of the HSC graft [205].

Stem Cell Expansion

The general protocol for regenerative therapies involves harvest of a patient's stem cells, expansion under in vitro conditions, and reintroduction into the patient to initiate regeneration [206]. However, stem cells such as MSCs show a decline in differentiation potential after prolonged passaging under in vitro conditions [207]. Therefore, the propensity of MSCs to differentiate spontaneously into a heterogeneous fibroblast phenotype on tissue culture plastic limits their clinical applications [206]. These limitations have prompted the development of nanoscale cell−material interfaces to direct the differentiation and growth of MSCs. In particular, the surface topography can regulate the balance of cell adhesion and cytoskeletal tension to affect the expression of MSC-specific markers such as CD63 [206].

In addition, a major bottleneck for stem cell therapy is the requirement for a large quantity of undifferentiated stem cells, especially to regenerate bulk tissues or organs [208]. This process is limited by the slow doubling times of stem cells, which can range from 36 h for human ESCs to 45 days for human HSCs [208]. Stem cells reside in specialized niches in vivo that promote their extensive self-renewal, so mimicking this environment ex vivo is a powerful approach to promoting stem cell expansion [209]. For example, the topography and texture of the basement membrane can be recapitulated on polyamide nanofibers and can promote the expansion of murine ESCs while retaining their differentiation potential [210]. The stem niche also contains multiple growth factors, including leukemia inhibitory factor and stem cell factor, which are sequestered in the ECM and can be presented to cells [209]. To mimic this microenvironment, growth factors can be covalently attached to nanofibers to prevent their degradation and can promote the expansion of stem cells such as human ESCs [211].

CONCLUSION

Nearly every human tissue has a nanostructure, and these nanostructures can influence cell activity such as cell adhesion, viability, and differentiation. Since the concept of nanotechnology was first proposed in 1959, this research field has developed rapidly for applications in regenerative medicine [212], especially in the fabrication of nanobiomaterials and nanoscale delivery systems. Nanomaterials can have a controlled shape, size, and topography that can be used to mimic the nanoarchitecture of tissue-specific ECM to direct cellular activity. Because of their small size and high surface area−to-volume ratio, nanomaterials can be internalized into cells to carry a large amount of growth factors and drugs. Nanomaterials with unique optical, magnetic, and electrical properties also can be used to track cells, stimulate cellular differentiation, or build functional tissue scaffolds. Depending on their composition, nanobiomaterials can be categorized as metallic-based, inorganic-based, carbon-based, polymeric-based, and biological protein−peptide based nanobiomaterials. These nanomaterials have been widely explored for potential applications in regenerating multiple types of tissues, including bone, muscle, cardiovascular, neural, cartilage, bladder, and skin, by creating nanopatterned tissue scaffolds or delivering biomolecules across tissue barriers. In particular, drugs, peptides, or nucleic acids can be delivered to a specific tissue, organ, or cell with high targeting efficiency, based on nanoscale delivery systems. In addition, nanomaterials can be used to track, deliver, and expend stem cells accurately, which are crucial factors for future clinical applications of stem cell−based regenerative therapies. We expect that advances in nanotechnology will contribute continuously to the field of regenerative medicine in the future.

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CHAPTER

30

Design Principles in Biomaterials and Scaffolds

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When tissue deficiencies occur as a result of trauma, disease, or congenital conditions, there is a desire to provide functional replacement tissues to the patient. The use of autografts is ideal when this option is available and is often applied for both soft and hard tissue defects with a broad variety of approaches, although donor site morbidity is a problematic consideration. In many cases, such donor tissue is not available and allotransplantation is favored, albeit with the frequently accompanying disadvantages of a limited donor supply and immunosuppressive therapy. In tissue engineering, a central concept is the application of a temporary biomaterial scaffold at the defect site to facilitate healing that will provide some restoration of functionality. If this scaffold is used to carry precursor cells or other features that may induce a functional healing, the outcome potential may be further improved. In designing such solutions for tissue repair and replacement, the parameters that define the scaffold must be selected and optimized to provide the best possible outcome. If cells are to be used, this adds further design (and regulatory) complexity. This chapter focuses on the design principles affecting degradable biomaterial scaffolds used in tissue engineering. Although acellular scaffold approaches are considered, the cellular component of seeded scaffolds will not be explicitly addressed; relevant considerations are covered elsewhere in this text.

FUNCTION AND APPLICATION-ORIENTED DESIGN OF BIOMATERIAL SCAFFOLDS

The objective for a given scaffold design is to meet the clinical needs for a specific application or set of applications. The better the understanding of the clinical need, including the underlying tissue physiology, disease pathology, and other impactful environmental parameters, the more appropriately the design parameters can be defined. As cellular and molecular biology knowledge have rapidly advanced and pathological processes are better defined, the ability to harness this knowledge in designing more advanced scaffolds has grown. The early use of temporary scaffolds in medicine was for simple mechanical support (e.g., sutures), whereas research efforts in recent decades have expanded the potential functional role that the scaffold can have, including the designed modulation of cell behavior and the incorporation of controlled release for bioactive agents.

Mechanical Support

Tissue loss and mechanical failure can be caused by various reasons, including disease processes, trauma, burn, surgical resection, and chronic inflammation. Biomaterial scaffold implantation may provide permanent or temporary mechanical support, hence maintaining the structural and functional integrity of the host tissue. In some reconstructive or cosmetic applications, the implanted scaffold role may be to improve aesthetics in addition to providing appropriate tactile or load-bearing behavior.

Mechanical support structures comprise a major part of the implantable biomedical device industry. Most of these devices are permanent and made from nondegradable metals and polymers. For instance, vascular stents are commonly nondegradable metallics such as nitinol, stainless steel, and cobalt-chromium [1]. Vascular grafts and abdominal wall meshes for hernia repair are often made from polyethylene terephthalate, polytetrafluoroethylene, polyurethane, and polypropylene [2,3]. In the orthopedic field, orthopedic screws and plates, staples, and other fixation devices are commonly stable metals [4,5]. However, each case, there has been substantial research and clinical

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investigation of degradable scaffolds [6–8]. The general cited advantages of using degradable scaffolds are (1) the potential for new tissue regeneration, (2) the reduced chronic stimulus for inflammation and ongoing foreign body response, (3) elimination of a nidus for infection risk once the material has degraded, and (4) the potential for reduced thrombotic risk and the elimination of anticoagulant therapy for blood-contacting scaffold applications. Despite these advantages, degradable scaffolds have not effectively displaced permanent scaffolds for a variety of reasons. Common factors limiting broader adoption include a higher risk for mechanical failure [9,10], increased early inflammatory response, and concerns with local tissue effects from degradation products and by-products [11].

It is common in the tissue engineering literature to find a hypothetical graph in which a hypothetical mechanical parameter is plotted versus time with three curves: scaffold, new tissue, and net construct (or combined). The scaffold curve slopes downward with time whereas the new tissue formation curve increases. Usually these curves are matched so that the net level of the mechanical parameter is maintained during the healing process. Of course, in reality this is often not the case; scaffolds remain longer than needed and potentially stress shield the tissue. Of greater concern is scaffold mechanical failure when the new tissue is insufficiently strong to take on the required load (Fig. 30.1). This is a serious concern in that such mechanical failures lead to morbidity and mortality that have limited the broader adoption of many degradable scaffold approaches, as indicated earlier. Also, such mechanical failure may be related to the underlying disease process in the treated population that may not be apparent in preclinical testing or in the application of such an approach in other populations. Therefore, in addition to selecting biomaterial scaffold designs with appropriate initial mechanical parameters approximating the host tissue, matching the degradation rate of temporary scaffolds to tissue integration and maturation is greatly important. In fact, sophisticated models seeking to capture the underlying physics and biology are under development [12].

At a cellular scale, a great deal of research in has examined how the microscale mechanical properties of biomaterial scaffolds can modulate cell behavior. Biomaterial substrate stiffness affects stem cell differentiation, including mesenchymal stem cells, induced pluripotent stem cells, and embryonic stem cells [13–15]. Many molecular pathways involved in this process have been described [16,17]. In addition to stem cell effects, substrate stiffness has been shown to affect primary cell phenotypes, including the macrophage and its polarization [18,19]. Macrophages are important cells involved in the foreign body response; thus, substrate stiffness also relates to the host tissue response to scaffolds. Researchers have shown that biomaterial stiffness modulates the adhesion, migration, and proliferation for other types of cells, as well [20].

Given the mechanical support functions that are defined for a specific clinical application of a scaffold, together with the increasingly appreciated effect that mechanical parameters can have on host response and cell behavior, selecting materials with suitable mechanical parameters is usually the first step in scaffold design. Generally, metals and ceramics have high stiffness and strength (Fig. 30.2A and B), as do many composite materials containing these two major categories of biomaterials for scaffolding. Between the two, ceramics have the disadvantage of the risk for brittle fracture. In comparison, synthetic polymeric materials and naturally derived materials are usually weaker in all major aspects. However, polymers and naturally derived materials have lower densities; thus, the differences in specific modulus and specific strength between them and metals and ceramics are not as large. Because most human tissues have stiffness values below the gigapascal range (Fig. 30.2C) [21], metals and ceramics are commonly used in hard tissue replacements (bone and teeth) and applications in which smaller scaffold dimensions are desired (e.g., coronary stents), whereas polymers and naturally derived materials are more often used for soft tissue substitutes.

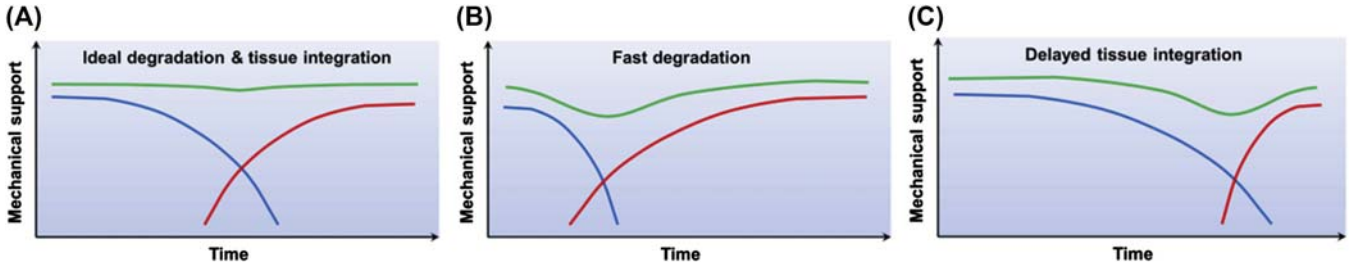

FIGURE 30.1 Mechanical support by scaffold–integrated tissue. The *blue line* indicates mechanical support by degrading scaffold; the *red line*, mechanical support by integrated tissue; and the *green line*, combined mechanical support by the scaffold–tissue combination. (A) Ideal matching between scaffold degradation and tissue integration. (B) The scaffold degrades too quickly and tissue generation is unable to compensate for some interim period, presenting a failure risk. (C) Slower scaffold degradation stress shields new tissue or prevents adequate cell migration and extracellular matrix (ECM) elaboration, leading to inadequate mechanical properties at a later time point.

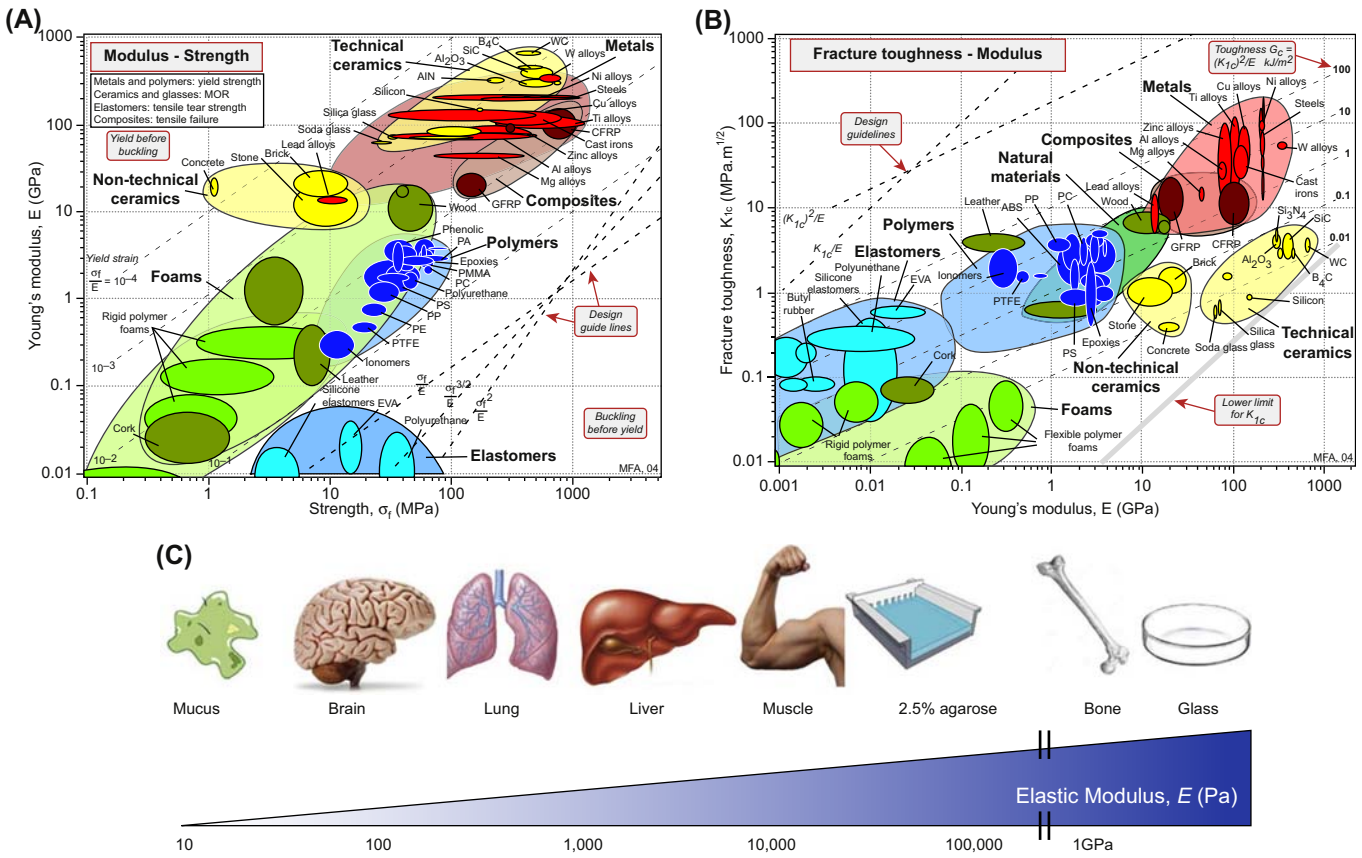

FIGURE 30.2 (A, B) Ashby chart of strength versus modulus. The "strength" for metals is the 0.2% offset yield strength. For polymers, it is the 1% yield strength. For ceramics and glasses, it is the compressive crushing strength, roughly 15 times larger than the tensile (fracture) strength. For composites, it is the tensile strength. For elastomers, it is the tear strength [22]. (C) Young's, or elastic, modulus of tissues [21]. *CFRP*, carbon fiber–reinforced thermoplastic; *EVA*, ethylene(vinyl acetate); *GFRP*, glass fiber–reinforced plastic; *MOR*, modulus of rupture; *PA*, phosphatidic acid; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PMMA*, poly(methyl methacrylate); *PP*, polypropylene; *PS*, phosphatidylserine; *PTFE*, polytetrafluoroethylene; *WC*, water cosolvent.

Degradation Profile

As noted previously, scaffold degradation is important for tissue integration and should be well-synchronized with the latter to maintain mechanical support in implantation sites. Aside from tissue integration, degradation can have a critical role in providing pathways for metabolite diffusion and angiogenesis, as well as the release of agents loaded into the material.

Degradation Mechanisms

The mechanisms at work in biomaterial scaffold degradation vary by material type. For polymeric biomaterials, the most commonly seen mechanism is hydrolytic cleavage of backbone bonds (ester, amide, urethane, and carbonate) in polymer chains by water molecules, producing oligomers and monomers with lower molecular weights and higher solubility (Fig. 30.3A) [23]. Enzymatic facilitation of such cleavage reactions can be an important factor in accelerating degradation in vivo, and the use of enzymes in degradation buffer solutions to study degradation in vitro is common. Polymer chains can also be cleaved by free radicals, irradiation, reduction reactions, and other reagents or stimulus, depending on the bonding mechanism [24–26]. Newer concepts in polymeric biomaterial degradation include increased solubility induced by side chain cleavage [27], self-immolating depolymerization [28], and the dissociation of supramolecular assemblies [29]. Metallic biomaterials are almost exclusively selected for their degradation (corrosion) resistance. However, a great deal of research has been focused on degradable metals such as magnesium and zinc alloys for use as temporary scaffolds in a broad variety of applications [30–32]. The corrosion process proceeds by coupled electrochemical reactions with electrolytes to produce oxides, hydrogen gas, hydroxides, or other compounds (Fig. 30.3B) [33]. A high concentration of Cl^- ions significantly accelerates

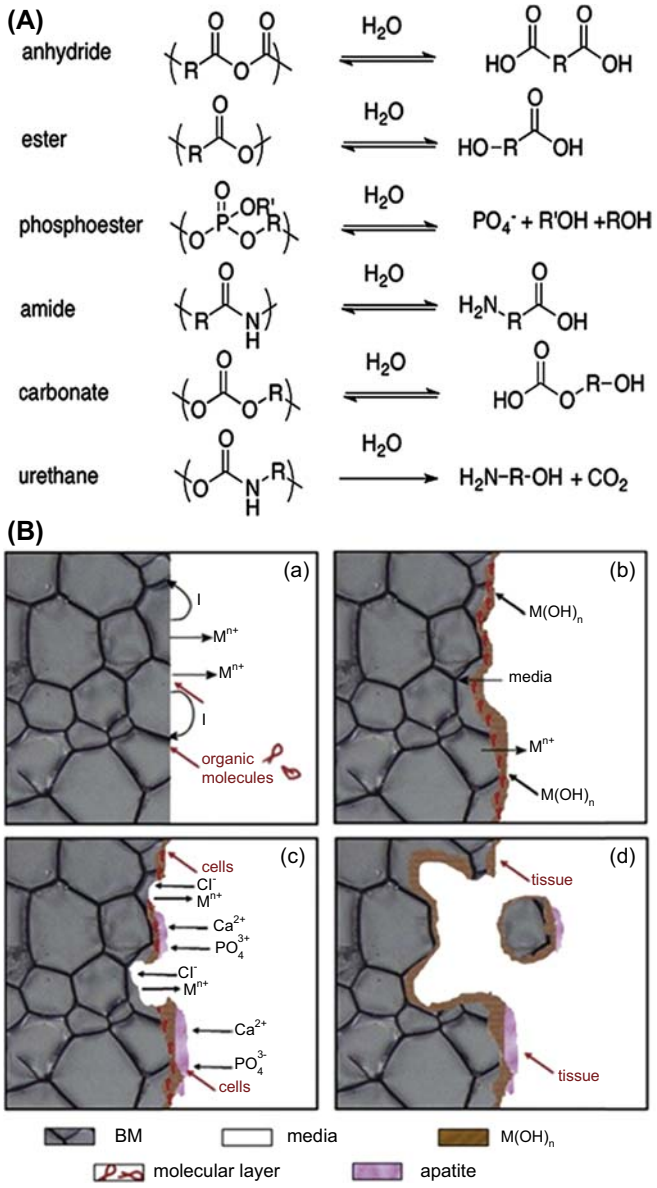

FIGURE 30.3 (A) Hydrolytically degradable linkages used in biomaterials. (B) Schematic diagram of biocorrosion at the biodegradable metal (BM)—medium interface [33].

the corrosion of metals. Cells are also subsequently involved in breaking down the metal substrate from the surface (Fig. 30.3B) [33]. The degradation of commonly used ceramic scaffolds (tricalcium phosphate, hydroxyapatite, and dicalcium phosphate) starts with the dissolution of CaP components, which is heavily affected by the solubility of the specific material. Various cell types (monocytes/macrophages, fibroblasts, and osteoblasts) are then involved in the degradation process by phagocytic mechanisms or via an acidic mechanism to reduce the microenvironmental pH that results in demineralization of the ceramic matrix and resorption [34]. For biologically sourced materials, specific enzymatic degradation has an essential role in degradation, and resistance to such degradation varies with the material composition, processing history and local tissue conditions.

Factors That Affect Degradation Rates

Several factors affect the degradation rates of biomaterial scaffolds, and these factors can be leveraged to modulate the degradation profiles of corresponding scaffolds. Most obviously, the molecular composition of the scaffold will dictate the degradation profile. For polymers with hydrolytically cleavable backbone bonds as introduced earlier, the bond type will heavily influence the degradation rate. For example, hydrolysis of anhydride bonds is

generally faster than ester bonds, which in turn is faster than amide and urethane bonds. Based on this effect, copolymerizing monomers that will result in different backbone bonds have been used to fine-tune the degradation rate of polyesters and other polymers [35]. The molecular environment around the labile bond and the relative density of labile bonds along the backbone are also important factors that can fine-tune the rate of degradation dramatically, despite maintenance of the same labile group. As a result, polyesters can vary as a class of polymer from very rapidly degrading to effectively nondegradable (e.g., polyethylene terephthalate). The hydrolytic cleavage of many of these susceptible bonds is acid catalytic and autocatalytic (degradation products of these bonds are acids); basic salts have been mixed with polyester substrates to slow down degradation whereas acidic monomers have been copolymerized purposefully to accelerate the degradation of polyesters [27,36]. Another obvious option in controlling polymeric scaffold degradation is to choose the initial molecular weight of polymer building blocks so that a greater or lesser number of cleavage events need to occur before solubility of the residual polymer is achieved.

For metallic scaffolds, innate factors affecting oxidation include the activity of the metal, phase organization (of alloys), impurities, the processing history, and the stability of the formed oxide layers [33,37]. For ceramics, the crystal type and degree of crystallinity are important parameters.

Enzymatic Degradation

Degradation on demand, or triggered degradation, is a concept receiving attention in the literature. For polymers, such triggers can include electromagnetic radiation, ultrasound, heating/cooling, or the delivery of a triggering molecule such as an enzyme. For metals, control of the oxidation/reduction reaction by the delivery of current is a ready means of control.

Looking at enzymatically triggered degradation, numerous examples of scaffolds employing this technique have been reported in which enzymes produced by the host tissue or delivered to the biomaterial scaffold accelerate degradation, particularly for naturally derived biomaterials and polymers. Matrix metalloproteinases, elastase, and other enzymes have been employed to cleave protein components in naturally derived biomaterials with high specificity, whereas enzymes such as hyaluronidase and glucanases cleave polysaccharides including hyaluronic acid, alginate, and chitosan. Some of these enzymes accelerate the degradation of synthetic polymers as well [38–42]. Segments of polypeptide, DNA, and other biomolecules have been deliberately covalently combined with synthetic materials at the molecular level or by physical mixing to provide cleavage sites for the composite scaffolds [43–45]. Because enzyme expression levels vary among tissues and some are specifically and temporally elevated in wound beds or sites of inflammation, they provide convenient local factors to be included and targeted in designing scaffold degradation profiles [46–48].

Surface to Volume Ratio

Scaffold morphology, particularly the ratio of the surface area to the volume, has a significant effect on the degradation of biomaterial scaffolds. Higher surface area ratios commonly lead to higher degradation rates of the scaffolds as a result of the greater access of backbone bonds to the surrounding aqueous environment and cofactors present in this environment. For example, electrospun nanofiber meshes made from certain polymers degrade faster than cast films with the same component [49,50]. However, in some important cases, porous structures can be associated with slower degradation [51]. This counterintuitive effect is postulated to result from the diffusion of catalytic reactants out of the scaffolds more readily in the porous case. Rapid transport facilitated by pores also serves to stabilize the pH in some Mg scaffolds, leading to a similar effect as that seen in some polymeric biomaterials [52,53]. Therefore, both opposing effects need to be considered when designing the porous structure in scaffolds. Methods of fabricating porous scaffolds are discussed in subsequent sections.

Surface Modification for Degradation Control

Another method for modulating scaffold degradation is to modify the surfaces. One mechanism is to change the surface hydrophilicity/hydrophobicity, thereby increasing or reducing water uptake that would affect the hydrolysis process. Generally, hydrophilic coatings increase degradation rates whereas hydrophobic coatings do the opposite [54–56]. For degradable metals, increasing corrosion resistance is usually the aim for surface modification, particularly when a given alloy is attractive for its processability, mechanical properties, or other considerations. Methods include simple polymeric coatings, molecular coatings that react with the surface oxide layer, plasma ion implantation, physical vapor deposition, thermal oxidation, and various electrochemical oxidation methods that have been widely applied to metallic biomaterials [56–60].

In considering scaffold degradation in general, many external factors such as mechanical load or irradiation can have a major impact on scaffold degradation rates. However, because the operational environment or processing requirements are often fixed, no further discussion of these factors will be made here.

Controlled Release of Bioactive Agents

Scaffolds may also serve as a reservoir for bioactive agent delivery including small molecule drugs, proteins, genes, cells and nanoparticles. Compared with the inherent physical and chemical features of scaffolds, the effects of released bioactive agents can extend beyond implantation sites to recruit or control circulating cells that are beneficial for tissue repair, and higher specificity can be achieved [61]. When two or more cargoes are loaded, corresponding release profiles may be programmable, which provides flexibility and can yield more desirable effects when orchestrated properly [62]. Logically, seeking synergy by combining the largely local benefits provided by the degrading scaffold with the diffusing effects from controlled-release agents has been a consistent direction explored in the effort to improve scaffold performance.

Many physical and chemical parameters of a scaffold affect the release profile experienced by the loaded cargo. Several of these factors overlap with factors regulating scaffold degradation, as degrading scaffolds open new channels for cargo diffusion. Some of the more important scaffold design parameters that influence the release of water-soluble agents are discussed subsequently.

Effect of Porous Structures

In diffusion-controlled delivery systems, porous structures provide an easier path for the molecules detaching from the material substrate to exit from the scaffolds and enter surrounding tissues, compared with diffusing through relatively dense material substrates. Therefore, easier access to pores and higher connectivity between pores facilitate faster soluble molecule release from a scaffold [63]. However, this theory does not always strictly apply because higher porosities may decrease the degradation rates of some polymer scaffolds, which would slow the accelerated delivery owing to the degradation effects.

At the molecular level, free volume will affect the diffusion rate. Taking hydrated hydrogel systems, for example, for gels with mesh sizes comparable to the size of the loaded agent, diffusion coefficients are decreased owing to steric hindrance provided by the cross-linked polymer chains [64]. Mesh sizes comparable to the size of the loaded agent increase the molecule diffusion path length compared with hydrogels with mesh sizes much larger than the releasing agent [64].

Affinity-Based Release

Affinity between the bioactive agent and the scaffold substrate affects the tendency of loaded molecules to permeate the material volume and approach the interface of the material−pore and materia−/tissue. Through transient interactions between the loaded cargo and the delivery scaffold, affinity-based systems (e.g., heparin-binding proteins for heparin, aptamers for nucleic acids, cyclodextrins for hydrophobic antibiotics) can minimize burst release while providing fine-tunable release profiles by attenuating diffusional release [65]. Related studies about affinity systems have shown that the addition of an affinity group to the therapeutic may be done so as not to affect the bioactivity of interest detrimentally, which supports the practicality of this strategy [66,67]. In addition, mathematical simulations have been developed to predict release profiles, which could be useful in predicting desired release profiles and designing release systems [65].

An alternative method to fabricate affinity-based release systems is to recognize a specific molecule selectively by the scaffold substrate known as molecular imprinting (Fig. 30.4) [68]. In molecular imprinting, the bioactive agents work as template molecules to create imprints in the polymer networks, which subsequently function as affinity binding domains [69]. Compared with nonaffinity systems, release retention was observed in imprinted systems [70]. Molecular imprinting systems would be advantageous for drug delivery because of their ability to sustain the release of a therapeutic agent, enhance the loading capacity, release the bioactive agents by in response to the stimuli intelligently, and enantioselectively load and release the eutomer (isomer of interest) [71].

On-Demand Release

The strategies mentioned earlier are effective passive methods to obtain sustained, prolonged release. However, it is often desirable to have a designed response to changing conditions as tissue healing or remodeling proceeds or to provide a more optimal delivery of an agent dependent on externally determined factors. More active, on-demand

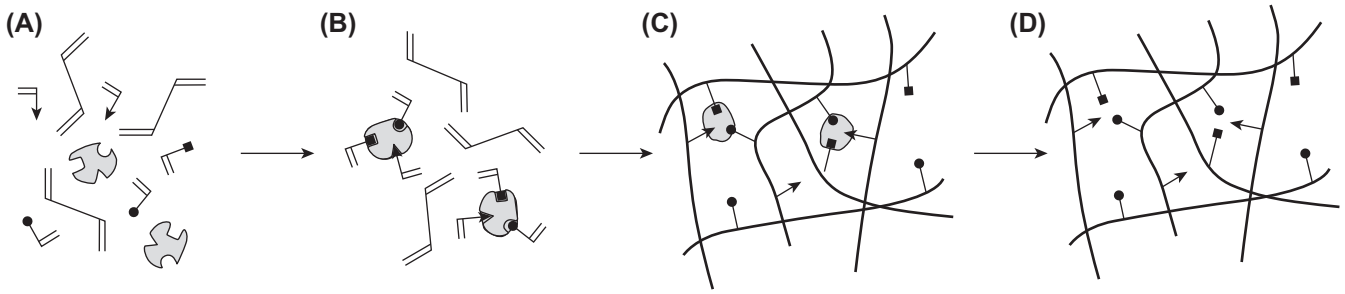

FIGURE 30.4 Molecular imprinting. (A) Solution mixture of template, cross-linking monomer, and functional monomers (triangles, squares, and circles). (B) Complex formation between functional monomers and template via covalent or noncovalent chemistry. (C) The formation of the polymer network typically via free radical polymerization. (D) Template removal step that leaves binding sites specific to the original template [68].

modulation of bioactive agent release can be achieved with on-and-off triggers. The most commonly used triggering mechanisms include (1) pH or temperature, (2) enzymes that cleave cross-linkers used to immobilize bioactive agents, or (3) drugs or ions that trigger the cleavage of an engineered substrate, resulting in the release of encapsulated cargoes [72–78]. Externally applied stimuli including light, electric or magnetic fields, and ultrasound can also modify scaffold structures or bioactive agent immobilization and regulate release [72,79]. The strategies introduced in this section can be applied to control the release of single bioactive agents and to program the delivery of two or more factors to obtain synergic effects [72,80,81].

Scaffold Morphology

As noted previously, scaffold morphology can greatly influence scaffold degradation, mechanical properties, and bioactive agent release. In this section, morphology is considered in terms of microscale pores and surface features, and progress in modulating scaffold morphological parameters is discussed.

Methods for Fabricating Porous Scaffolds

Conventional fabrication methods to generate porous scaffolds commonly used in tissue engineering include temperature-induced phase separation, salt leaching, gas foaming, electrospinning (for polymers and natural products); gas injection through the melt, gas foaming, plasma spraying, sintering, space holder methods (for metallic scaffolds); and dry-pressing, extrusion, and slip-casting (for ceramic scaffolds). The literature provides many excellent reviews covering these approaches and applications with materials processed in this manner [82–86]. Beyond these methods, newer technologies allow more design control over the scaffold geometry.

Solid free-form fabrication (SFF) technologies are the most important advances made in scaffold fabrication [87]. Among SFF, three-dimensional (3D) printing fabricates 3D structures by inkjet printing liquid binder solution onto a powder bed (Fig. 30.5A). Fused deposition modeling deposits molten thermoplastic materials through two heated extrusion heads with a small orifice in a specific lay-down pattern. The basic concept of stereolithography is to control the polymerization spatially of photocurable resin in a 2D pattern and to extend it to 3D by repeating in-plane polymerization in a layer-by-layer fashion (Fig. 30.5C). Selective laser sintering and melting employs a laser to scan the surface of powdered polymer or metal particles in a specific 2D pattern to sinter by heating them above the melting temperature. 3D plotting or direct-write bioprinting extrudes a viscous liquid material from a pressurized syringe into a liquid medium with matching density and deposits the materials in one long continuous strand or in individual dots to create desired 3D shapes (Fig. 30.5B) [87–91].

Anisotropic and Gradient Scaffolds

Anisotropy and gradient features are widely found in human tissues and organs, including the interfaces between tissues, such as the bone–cartilage interface. This morphology has important implications for the mechanical and biological behavior of these tissues. Therefore, it is interesting to fabricate anisotropic and gradient biomaterials as scaffolds to guide regeneration in a manner that provides greater biomimicry of the native state. Anisotropic and gradient cues include composition, pore structure, stiffness, and fiber orientation. A variety of scaffold processing methods have been developed. Listed here are several representative examples for both methodologic and therapeutic effects.

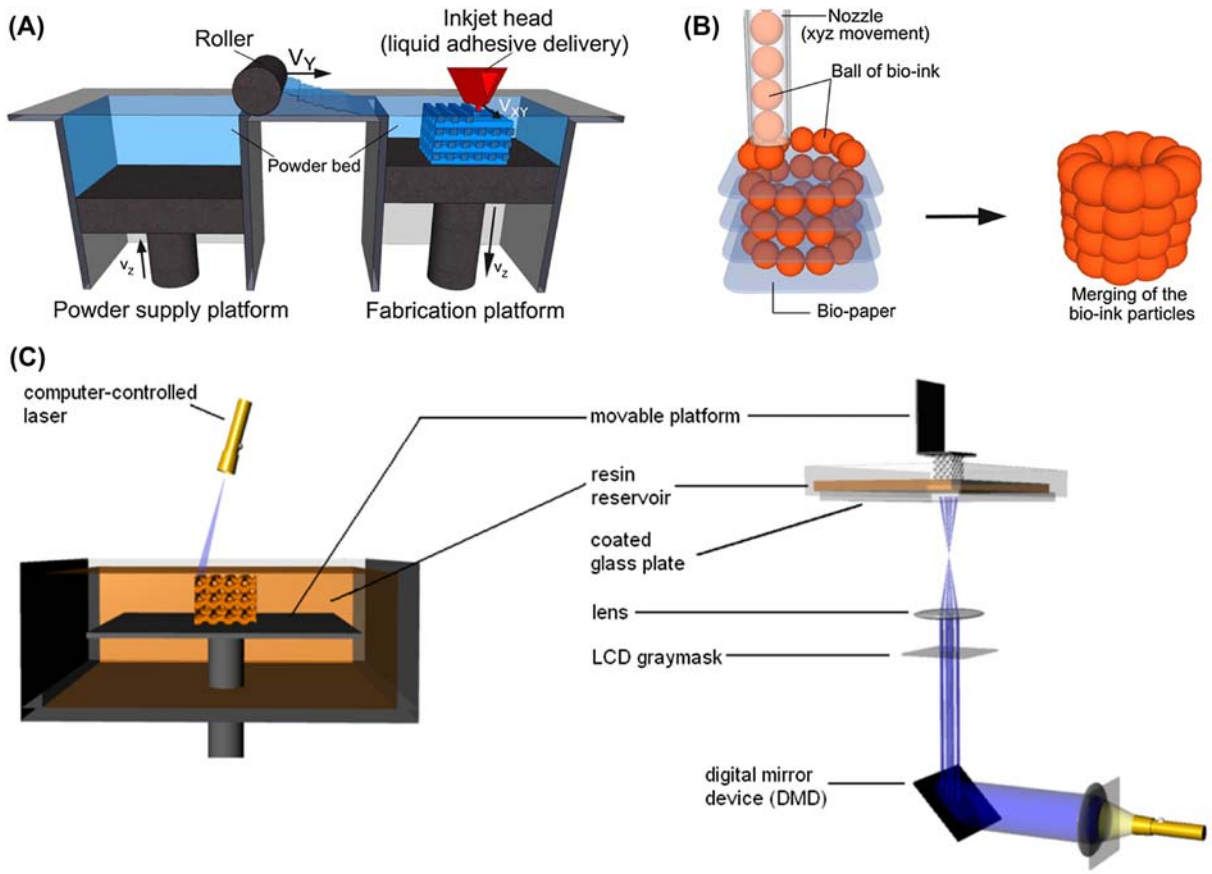

FIGURE 30.5 Schematic illustration of (A) three-dimensional (3D) printing, (B) 3D plotting/direct-write bioprinting, (C) stereolithography [88–90]. LCD, *liquid* crystal display.

Concentration gradients of bioactive molecules such as growth factors are often desired to drive spatially distinct biological responses to a scaffold. Such gradients have been created using microfluidic platforms or allowing a limited period of diffusion from a reservoir connected to one edge of a scaffold. In a case where platelet-derived growth factor was differentially loaded, tissue invasion depth and blood vessel density increased with the magnitude of the gradient, whereas cortical neurites showed sensitivity to the magnitude of an insulin-like growth factor-1 concentration gradient [92,93]. By controlling the cross-linking density, a gradient modulus was achieved in a polyethylene glycol (PEG) hydrogel. The human osteoarthritic chondrocyte number and phenotype were maintained in regions with a lower storage modulus compared with stiffer regions [94]. A modified one-step gravity sintering method was used to distribute pores with gradient sizes in calcium polyphosphate scaffolds, which induced greater osteoblast differentiation and mineralization in gradient calcium polyphosphate scaffolds in contrast to homogenous calcium polyphosphate scaffolds [95].

Creating thermal gradients when fabricating temperature-induced phase separation scaffolds was effective in arranging unidirectional porous structures and induced anisotropic cell attachment, proliferation, and migration [96,97]. Aligned fibrous scaffolds could be prepared by 3D printing and electrospinning. These unidirectional features have been employed in organizing new tissues including nerve, cornea, tendon, and muscle [98–100]. Applying similar processing methods on both x and y directions could mimic alternating orientating cells and ECM such as that in laminar cardiac tissues [101,102].

Surface Feature Manipulation

It is widely known that surface features and patterns can have a significant influence on cell attachment, locomotion, survival, and differentiation [103,104]. However, discrete patterns can be challenging to fabricate across the surfaces of 3D scaffolds using many of the most common fabrication techniques. Less discrete control of the surface is

more readily achieved, effectively varying the roughness, to affect responses of adhering cells [105,106]. Appropriate creation of microsurface or nanosurface structures can either promote or reduce cell adhesion [107,108]. In addition, cell migration can be guided by micrometer-scale roughness and hydrophilicity gradients [109,110]. Using specific interactions with adsorbed proteins and cell surface receptors, cell migration can be modulated [111].

Injectable Scaffolds/Controlling Morphology In Situ

Traditionally, biomaterial scaffolds are formed in vitro before implantation. Technologies have allowed the minimally invasive delivery of scaffolds and the formation in situ of desired microstructures, which reduce the risk for invasive surgical procedures [112]. In addition, in situ–formed pores would allow cell infiltration before significant scaffold degradation, which may promote tissue integration.

Metallic scaffolds including coils and stents can be compressed with large deformation ratios to allow packaging on catheters for guided delivery. After deployment in targeted regions, these scaffolds can either revert to their original shape with high elasticity or be shaped by catheter-associated mechanisms such as inflatable balloons. Porous ceramic and polymeric scaffolds can be delivered by injection as solution precursors mixed with porogens [113,114]. As the biocompatible solvents absorb and the porogens dissolve in vivo, the result is porous structures. An alternative method to forming porous hydrogels in situ is to bind micrometer building blocks covalently via self-assembly in the tissue [115]. Elastic, high water–content hydrogels are squeezed through syringe needles and partially regain their porous structure in vivo [116,117].

Traceability and Imaging

Common to preclinical biomaterial and medical device evaluations is the recovery at different time points of implanted scaffolds from experimental animals or patients for subsequent histological, proteomic, and genetic assessments. Valuable information has been extracted by employing this paradigm. However, real-time monitoring options for the scaffold or tissue environment are limited and constrain the capacity of researchers and clinicians to monitor scaffold performance and sense potential negative events such as mechanical failure and detailed aspects of the biological response. Furthermore, as the tools to manipulate the status of smart, responsive biomaterials continue to expand, the desire is increasing for parameters that reflect scaffold status at given postimplantation time points.

Tracking scaffold material with noninvasive imaging techniques including magnetic resonance imaging, ultrasound, and computed tomography scanning is favored. Essential to scaffold tracking is the ability to differentiate the biomaterial signal from the tissue background. Different signal generation mechanisms are involved in these imaging techniques, with contrast originating from differences in parameters such as water content, radio opacity, stiffness, density, and magnetism. Contrast can be specifically created by adding contrast agents during scaffold formation [118–121]. However, the potential toxicity of contrast agents needs to be considered [122]. Greater contrast properties for the scaffold provide for improved resolution in the imaging techniques to reveal greater structural detail. Although not considered in this chapter, the labeling of loaded cells is commonly pursued in the context of tissue constructs [123].

SAFETY AND BIOCOMPATIBILITY REQUIREMENTS FOR BIOMATERIAL SCAFFOLDS

Achieving the desired level of functionality for a given medical application is obviously central to assembling the design objectives for a biomaterial scaffold system. However, the safety of such a system must explicitly be demonstrated as the pathway to clinical application is traversed. Several general concerns will apply to all scaffolds, whereas other considerations will be specific to the given application. The governing regulatory body (e.g., the U.S. Food and Drug Administration) will ultimately dictate the specific testing required for progression to early-stage clinical evaluation. Biomaterial scaffolds are medical devices by definition, and as the World Health Organization has pointed out in its guidelines: (1) absolute safety cannot be guaranteed, (2) the consideration of a given device's safety is a risk management issue, (3) safety is closely aligned with device effectiveness and performance, (4) safety must be considered throughout the life span of the device, and (5) the assurance of safety requires shared responsibility among the stakeholders [124]. As specifically addressed in International Standards Organization Document 10993, some of the principal considerations in demonstrating device safety include local and systemic

toxicity, carcinogenicity, pyrogenicity, sensitization, potential for infection, hemocompatibility, and adverse foreign body responses. Furthermore, because the scaffold system is biodegradable, the evaluation of biodegradation products both in vitro and in vivo must be explicitly addressed.

Infection and Sterilization

Bacteria can adhere to biomaterial scaffolds before, during, and after the implantation procedure. Although completely eliminating the possibility of bacterial contamination before implantation is impossible, major sterilization protocols including radiation, ethylene oxide (EtO), hydrogen peroxide, or steam can be designed to achieve a recommended sterility assurance level (SAL) (usually a risk of one nonsterile device in a million) [125]. The scaffold thus needs to be compatible with at least one of the sterilization methods and the design processes should reflect consideration for sterilization step. The various methods each have trade-offs in terms of their impact on the underlying material, expense, and integration with the manufacturing process. Specifically, the sterilization procedure may affect material performance by the same mechanisms used to kill bacteria, e.g., each of these sterilization methods can decrease the molecular weight of PEG-based polymers [126]. For scaffolds made from natural products (such as proteins or polysaccharides) and those containing naturally derived bioactive ingredients, EtO, heating, and radiation may denature and alter the structural or functional components of the scaffold. Although metals and ceramics are more stable compared with polymer and naturally derived materials, the labile versions of these materials can be more sensitive to some of the sterilization methods. For all of the scaffold types, because degradability is a feature, considerations must be given to packaging and storage methods, including the determination of shelf-life under prescribed conditions.

Across all implanted medical devices, the incidence of device-centered infections is much higher than the SAL, which has led to the conclusion that most infections occur as a result of exposure to bacteria during the implantation procedure or after device placement (Fig. 30.6). An attractive feature of biodegradable scaffold systems is the limited implant period, because the scaffold is replaced by native tissue, which removes the nidus for infection that remains with nondegradable devices. Current strategies that have been explored to reduce the potential for infection in nondegradable devices generally apply to scaffolds as well. These approaches fall into three general categories: (1) surface modification to impart resistance to bacterial adhesion [127,128], (2) loading of antibiotic agents into the biomaterial for controlled release [129,130], and (3) modifying or otherwise designing the surface to encourage

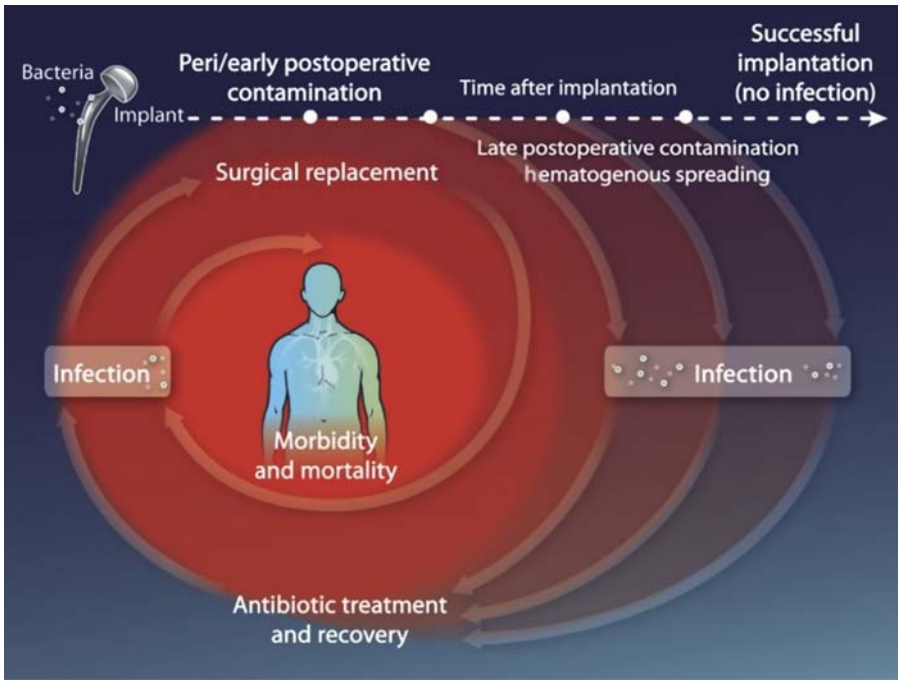

FIGURE 30.6 Patient risk factors for developing a biomaterial-associated infection. Revision surgery patients are at greater risk than primary surgery implant patients, whereas the risk of an implant or device becoming infected hematogenously decreases with time after implant placement due to more extensive host tissue integration [132].

rapid coverage and integration with host tissue, thus minimizing the likelihood of further exposure to and colonization by planktonic bacteria [131]. Because the scaffold design generally seeks to integrate and be replaced with native tissue, the latter approach is particularly attractive, although controlled antibiotic release in the early period may also be attractive.

Toxicity

Major components of scaffolds are not present in the human body (e.g., polycaprolactone) or at unnaturally high concentrations (e.g., high dosage of magnesium in magnesium scaffolds). These components and corresponding degradation products may induce acute, subacute, or chronic toxicities, especially with allo- and xeno-sourced products [133]. Pyrogenicity, genotoxicity, carcinogenicity, reproductive, and developmental toxicity are the main risks of scaffold toxicity.

Endotoxins are abundant in the environment and have high affinity to various biomaterials; they are the major factors that cause pyrogenicity [134]. In addition, endotoxins have strong proinflammatory effects. Different methods have been developed to lower the endotoxin level in scaffolds [135,136]. Resin monomers, glass ionomers, graphene oxide nanosheets, and other substances have been shown to be genotoxic [137–139]. On the other hand, much research has been focused on testing carcinogenicity in chemicals and small particles and has identified a variety of risky species that are dissolved components or wear debris of biomaterial scaffolds [140,141]. Improvements in scaffold design could mitigate the carcinogenic potentials, e.g., lower the possibility of generating debris production [142]. Organ-on-a-chip and laboratory-on-a-chip technologies have been employed to fabricate medical-device-on-a-chips as in vitro models for medical device toxicity tests [143].

Hemocompatibility

Scaffold hemocompatibility is a concern in the acute period when the device placement procedure involves some level of blood contact, and more chronically for scaffolds that will experience ongoing exposure to blood, such as with stents, cardiac valve scaffolds, or vascular grafts and patches. For all concerns regarding blood-contacting materials, the potential exists for the material to induce hemolysis, complement activation, and the formation of thrombus or embolus. Hemolysis can result from the material properties or the induced flow path created by a scaffold, such as high–fluid shear regions. The characterization of hemolysis for materials and devices is well-described in standards and in the literature [144,145]. Complement activation is generally related to surface properties; guidance exists to assess the activation of this immune system pathway [146–148]. Like hemolysis, thrombogenicity depends on both the material properties and the blood flow pathway near the scaffold surfaces. The status of the patient's blood is also a consideration, because anticoagulant and antiplatelet medications can be prescribed to reduce the safety risks associated with thrombosis.

Thrombogenicity remains a major cause of scaffold failure for blood-contacting biomaterial scaffolds, including vascular grafts and stents [149,150]. Thrombi formed on scaffolds may occlude vessels or embolize and lead to life-threatening ischemia in distal tissues (e.g., pulmonary emboli or embolic stroke) [151]. Because the coagulation pathway and platelet adhesion are initiated on artificial surfaces by protein adsorption, the design of scaffold surface properties to alter this adhesion process to reduce thrombogenicity has long been a focus of research in the biomaterials community. Common approaches include those that seek to reduce overall protein adsorption by increasing surface hydrophilicity, presenting zwitterionic groups, attaching specific bioactive molecules, or manipulating surface roughness. Surface attachment of the anticoagulant heparin or heparin analogs is one of the most widely employed strategies [152,153]. Surface modification with zwitterionic polymers such as phosphoryl choline or sulfobetaine derivatives can effectively reduce protein adsorption; it has been shown to reduce thrombogenesis for a variety of underlying surfaces [154–156]. Layer-by-layer assembly has also used to create antifouling surfaces [157]. Rough surfaces generally increase the potential for platelet deposition [158]. Therefore, smooth and even lubricious scaffold surfaces have been employed to reduce thrombogenesis [159,160], although an alternative approach of presenting rough surfaces to encourage rapid tissue integration has been used in chronically placed nondegradable devices [161,162]. With the theme of tissue integration central to scaffold placement, the development of an endothelial layer over the blood-contacting surfaces is an attractive way to reduce the ongoing risk for thrombosis and thromboembolism. This is a common vision for blood vessel, cardiac valve, and stent scaffold design. Such endothelialization may be encouraged by specific surface ligands or the controlled release of bioactive agents, as well as endothelial cell or endothelial progenitor cell seeding or recruitment from the circulation or nearby tissue [163–165].

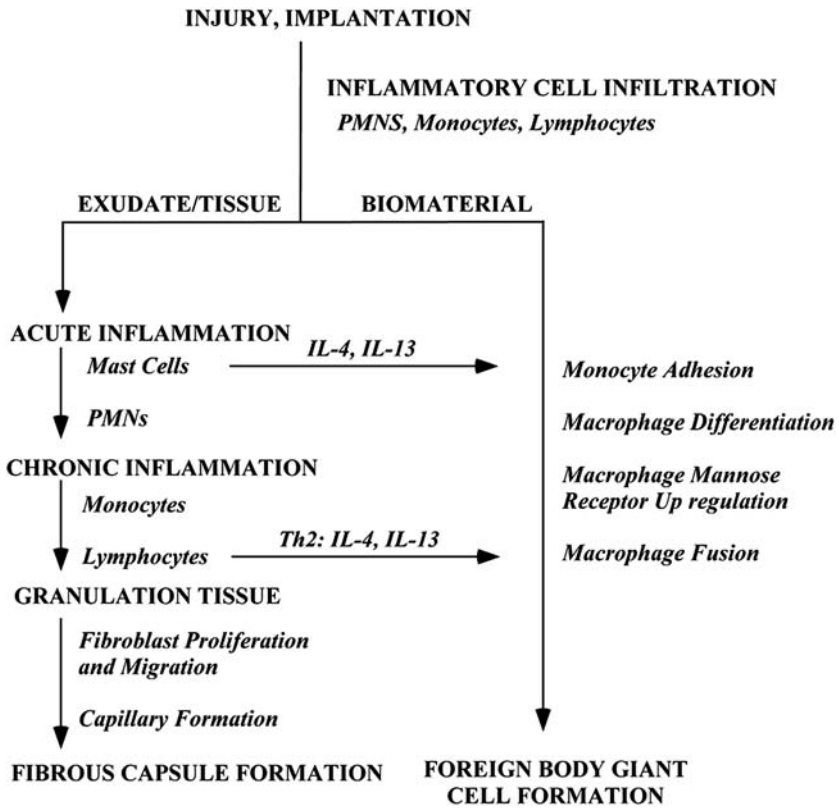

FIGURE 30.7 Sequence of events involved in inflammatory and wound healing responses leading to foreign body giant cell formation. This shows the potential importance of mast cells in the acute inflammatory phase and T helper 2 (Th2) lymphocytes in the transient chronic inflammatory phase with the production of interleukin (IL)-4 and IL-13, which can induce monocyte and macrophage fusion to form foreign body giant cells. *PMN*, polymorphonuclear leukocytes [167].

Foreign Body Response

Implantation of biomaterial scaffolds induces inflammatory and wound healing pathways, which result in a common response if the material is synthetic and nondegradable. Similarities of the response across materials and implant locations has led to the designation of these sequelae as the "foreign body response" (Fig. 30.7). Degradable scaffolds will ultimately remove a central feature of the chronic foreign body response (the implant immediately surrounded by encapsulating fibrous tissue), but during the degradation period of the scaffold, the response is roughly equivalent, although ultimately with more active phagocytosis by macrophages in the surrounding tissue. Of note, with materials based on decellularized tissue, this response outcome may follow a different pathway that has been termed "constructive remodeling," with important differences in the cellular and molecular components [166]. From a safety perspective, the extent of inflammation and fibrosis in the area of scaffold placement is a concern in terms of how this response may lead to failure of the device or inhibition of the device's function. Also of concern is the variability that may occur with this response such that in some patients a more vigorous inflammatory response may lead to accelerated scaffold degradation and early mechanical failure or other morbidity such as local pain and swelling. Readers are encouraged to refer to reviews for the latest concepts regarding foreign body responses [167–170].

Because macrophage behavior is a critical determinant in the direction of the foreign body response, there has been attention to examining how macrophage behavior can be influenced by scaffold topography, stiffness, surface chemistry, and naturally derived bioactive components [19,167,171,172]. Of interest to the biomaterial community are strategies that may serve to influence the foreign body response by modulating macrophage behavior. One approach is to passivate scaffold surfaces in an attempt to render the material substrate "invisible" or neutral to macrophages. Ultralow-fouling zwitterionic hydrogels resisted macrophage adhesion and capsule formation for at least 3 months in a mouse subcutaneous model [173]. In another study, triazole derivatives of alginate were identified to modulate immune cell populations at the surface of hydrogels made from these molecules, specifically

macrophages, in a manner that inhibited their activation and disrupted fibrotic processes, leading to the mitigation of foreign body response in nonhuman primate models [174]. A second strategy has been to polarize local macrophages actively toward an M2 phenotype (a phenotype associated with the promotion of tissue repair and regeneration in contrast to the proinflammatory M1 phenotype [168]). It was widely reported that a decellularized tissue-based material alternative referred to as ECM-based products can induce M2 polarization of macrophages. Thus, aside from fabricating scaffolds directly from ECM products, efforts have been made to produce composites of scaffolding material, usually synthetic polymers, and ECM-derived components as additives [168,175,176].

MANUFACTURABILITY

Even at the earliest stages of the design process for biomaterial scaffolds, it is worth considering how scaffold production ultimately might be scaled up in a manner consistent with good manufacturing practice to ensure stable quality. More broadly, translational and regulatory challenges from the manufacturing perspective will need to be addressed if the scaffold is to move toward a clinical impact, and early identification of design limitations in this area may allow the implementation of an approach with a greater likelihood of success.

As introduced earlier, selecting the scaffold material is often the first step in scaffold development. Regulatory agencies focus on application-oriented pathways in which a specific type or composition of material is approved for one application (device) at a time and reevaluation is needed for a different application. The selection of widely used materials in approved devices such as poly(lactic-co-glycolic acid), stainless steel, and hydroxyl apatite can be attractive because their safety as components in existing products has been demonstrated and general sterilization and biocompatibility protocols have been addressed in a manner that may greatly reduce the material-associated risk for the new application. Furthermore, supplier and manufacturing considerations, from raw material sourcing to material synthesis, and even scaffold formation may have been addressed successfully. Taking polymeric biomaterials as an example, despite the exploding diversity of material designs reported in the literature and the great advancement with controlled polymerization, a limited set of degradable polymers is still commonly implemented owing to practical considerations regarding regulatory approval, costs, and manufacturability. Although newer designs for degradable polymers may possess a better-controlled structure and molecular weight, enabling the integration of powerful biological and imaging functions, the synthesis and processing steps may be markedly more complex, require more strictly monitored controls and systems, and require a more extensive approval pathway compared with a less effective but still functional material. Some of these obstacles may be overcome by modifying laboratory fabrication protocols and considering trade-offs in complexity or benefit early in the design and evaluation process.

With rapid advances in SFF and the attractiveness of combining such technology with patient-specific imaging and even personalized medicine, the promise is presented of better designs suited for individual patient needs. One could imagine controlling the material, morphology, and bioactivity in a patient-specific manner. This will be an area of substantial investment in coming years, and if the patient benefits are great enough compared with nonindividualized solutions with simpler materials, the cost of implementing such technologies in a regulatory-compatible and economically attractive manner would be justified.

SUMMARY

The use of temporary biomaterial scaffolds for regenerative medicine approaches to tissue failure provides a means to support the transition from a synthetic, externally generated “bandage” to an autologous, functional tissue outcome. The potential to mediate a complete and effective transition remains elusive for many tissues and disease states. For the team designing such regenerative medicine solutions, the considerations for the scaffold are many, and there is much more potential for the scaffold than to serve as a well-designed physical support that appropriately transfers load to ingrowing tissue. As noted in this chapter, the team has an increasing array of tools to control the degradation process, design for tissue integration, deliver bioactive agents in an appropriate temporal fashion, and use these design options to meet specific hypothesized needs for the tissue and patient group in question. Ultimately, scaffold design considerations must fit within a much broader array of considerations that will touch upon many other topics covered in this text. As with many of those other topics, the field is dynamic, challenging, and ripe with the opportunity for new investigators to have an impact on the future of regenerative medicine.

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CHAPTER

31

Proteins Controlled With Precision at Organic, Polymeric, and Biopolymer Interfaces for Tissue Engineering and Regenerative Medicine

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WHY THE NEED FOR PRECISION CONTROL OF PROTEINS AT INTERFACES IN TISSUE ENGINEERING AND REGENERATIVE MEDICINE?

Evolution has crafted proteins to communicate information in biological systems with precision and accuracy. Living biological systems present the correct protein in the correct orientation and conformation to deliver protein signals. Such protein signal delivery typically occurs at cell surfaces or on the surfaces of extracellular matrix proteins. In areas where there should be no protein signals, nature uses nonfouling strategies to prevent nonspecific interactions. Overall, this leads to high signal-to-noise: the specific signal is optimally delivered and the "noise" from nonspecific proteins is minimized.

An example of such precision protein signaling that occurs in living systems is seen with bone morphogenetic proteins (BMPs), a family of proteins that have been evolutionarily important for over a billion years. BMP proteins are involved in biological signaling for skeletogenesis, limb regeneration, body axis determination, tissue morphogenesis, germ layer direction, and cell-fate determination, among other signal pathways [1]. BMPs affect so many different biological processes, and these processes are so critical to organism development, that the signals must be delivered with almost perfect specificity and temporal control. It has been found that BMP signals delivered after interaction with type I collagen show enhanced bioactivity compared with the soluble protein [2]. A similar observation was made with osteopontin noncovalently immobilized on type I collagen [3]. Important for this precision signaling are the specific BMP proteins involved, accessibility of the active site on the protein (molecular orientation and conformation), protein concentrations, concentration gradients and temporal persistence of the signal delivery.

In contrast, biotechnological approaches, particularly using nonbiological materials, do a poor job of delivering protein signals. Proteins are often nonspecifically adsorbed to surfaces with no control of orientation and conformation, and sometimes little or no control of which proteins adsorb. For example, consider cell culture. Billions of tissue culture polystyrene (TCPS) plates are sold each year for use in most every area of cell biology, biotechnology, and medicine. The medium pipetted into these plates is frequently supplemented with fetal bovine serum or related complex biological fluids that contain hundreds of different proteins. The TCPS surface interacts with some complex mixture of proteins that reach the surface, adhere (absorb), and are not displaced by other proteins. These proteins can adsorb with their biologically relevant faces up, or down, or in some intermediate orientation. Just a small fraction of the proteins that adsorb to the surface are integral to cell attachment and growth (for example, fibronectin or vitronectin). Furthermore, the multiprotein surface can be highly denatured, in a native conformation or partially denatured. Couple this lack of precision in control of the protein surfaces with irreproducibilities caused by animal-to-animal variation in the proteinaceous components of the media and

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it is surprising that we have any reproducibility at all in our cell technologies. Where the absence of precision control becomes particularly evident is when we attempt to control cell differentiation at surfaces. TCPS is poor at preventing phenotype drift in cell lines on culture plates and poor at inhibiting stem cells from differentiating where we want to culture and replicate those cells in their stem-like state.

For implanted medical devices and tissue engineering scaffolds made of synthetic materials, these materials adsorb complex, nonspecific protein layers immediately after implantation. It has been hypothesized that such layers (many proteins in many states of orientation and denaturation) are responsible for the collagenous encapsulation (foreign body reaction) observed in most cases with synthetic material implants: the macrophages interrogating the implant do not recognize such multiprotein, multiconformation surface films as part of normal physiology and thus trigger an encapsulation reaction to isolate the implant from the body.

Nonspecifically adsorbed proteins have also been implicated in the thrombogenicity of synthetic surfaces in blood. Fibrinogen on surfaces has been implicated as the principal activator of blood platelets. In a study on fibrinogen adsorbed to a series of polyurethane polymers of different compositions, it was noted that the amount of adsorbed fibrinogen correlated directly with the platelet adhesion for most of the polymers [4]. However, there were some significant outliers and it was hypothesized that these were related to proteins in different states of denaturation on the different surfaces.

Decellularized tissue scaffold-based approaches to tissue engineering may also benefit from the controlled use of surface proteins. The processes used for decellularization may damage the recognizability of the proteins comprising these scaffolds. After decellularization, treating the scaffolds with signaling proteins can restore biospecificity [5] while taking advantage of the biocompatibility, biodegradability, unique pore geometries, and good mechanical properties of such scaffolds. Also, new functionality not present in nature can be added to the scaffolds.

SURFACE ANALYSIS AND ITS ROLE IN THE PRECISION DELIVERY OF BIOLOGICAL SIGNALS

Many proteins are roughly 5–10 nm in size. The recognition sites on proteins (protein-ligand binding sites) responsible for biological signaling are closer to 0.5–1 nm in dimension. These dimensions are appropriate for the analysis depths of many contemporary surface analysis methods (Fig. 31.1). Furthermore, preparing the

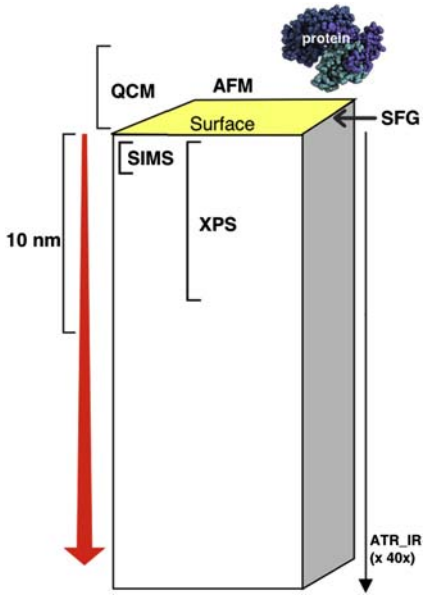

FIGURE 31.1 Consider a surface interacting with a protein. A typical protein molecule (about 5 nm) is shown to establish the size scale. Techniques such as sum frequency generation (SFG) and atomic force microscopy (AFM) are highly surface localized. Quartz crystal microbalance (QCM) methods, especially with dissipation monitoring can measure the protein and its viscoelastic response in the surface zone. Secondary ion mass spectrometry (SIMS) penetrates 1–2 nm into a surface or into a protein. X-ray photoelectron spectroscopy (XPS), also called electron spectroscopy for chemical analysis (ESCA), looks approximately 8 nm into a protein or surface. Other methods such as attenuated total reflection infrared (ATR_IR) look microns into a surface, provide vibration spectroscopy information, and are still considered surface analysis methods.

substrate surface for the immobilization of proteins often involves complex chemical modifications so that specialized tools are needed to monitor complex chemistry modifications. Protein–biomaterial interfaces are typically complex, and validation that a chemistry or surface organization is indeed as might have been envisioned in a Powerpoint image is essential for reproducibility and to achieving predicted functionality. Modern surface analysis methods offer exceptional abilities to probe surfaces chemistries and to compare hypothesized surface organizations with real-world data [6]. A few of the more powerful and versatile methods will be briefly described here. Their application to specific chemistries for delivering biological signals will be elaborated upon in the section on Techniques and Technologies for Precision Immobilization at Surfaces.

A SHORT REVIEW OF KEY SURFACE ANALYSIS METHODS AND SUPPORTING TOOLS

X-ray Photoelectron Spectroscopy

X-ray photoelectron spectroscopy (XPS) can determine compositional information in the uppermost 8–10 nm of the substrate surface [7–10]. XPS has been used to verify the quality (identity and contaminants) of the substrates and quantify protein adsorption. X-rays bombard the sample, and electrons from the atoms' core and valance bands, called photoelectrons, are ejected and analyzed [7]. The kinetic energy (*KE*) of the photoelectrons is measured and the binding energy (E_B) of the photoelectrons is calculated using the X-ray photon energy, which is a fixed, known energy. For example, for aluminum Kα, a common X-ray source, the X-ray photon energy ($h\nu$) is 1486.6 eV. The binding energy is given by: $E_B = h\nu - KE - \phi$, where the work function (ϕ) is the minimum energy required to eject a photoelectron into the vacuum from the highest occupied energy level. The work function can be calibrated using a clean bare gold sample and remains constant as long as the spectrometer is maintained in ultrahigh vacuum. Manufacturers of instruments usually provide a value for the work function. Insulating samples require charge neutralization using a low-energy (<20 eV) electron flood gun.

XPS provides both quantitative information (atomic percentage) and chemical-structural information (molecular bonding). The atomic percentage of each element is governed by the photoelectron yield at a particular binding energy. Elemental compositions (atomic percentages) are calculated from the measured peak areas by using appropriate instrumental sensitivity factors and accounting for instrumental conditions such as the photoelectron takeoff angle. For protein adsorption, the nitrogen atomic percentage can be monitored because the protein typically contributes a larger nitrogen signal compared with the substrate [10]. The binding energy of the photoelectrons depends on the type and environment of the atom. Therefore, high-resolution XPS spectra can be used to determine the type of chemical species (hydrocarbon, fluorocarbon, metal oxide, etc.) present in the sample surface.

Time-of-Flight Secondary Ion Mass Spectrometry

Time-of-flight secondary ion mass spectrometry (ToF-SIMS) can be used in the static mode to generate a mass spectrum or a two-dimensional (2D) chemical image of the outer 2 nm or so of a surface [11]. In the dynamic mode, ToF-SIMS can be used to generate depth profiles or 3D chemical images of a sample. In a ToF-SIMS experiment, surfaces are bombarded with a pulsed, primary ion beam that sputters atoms, molecular fragments, and entire molecules from the outermost surface. Most of sputtered material is neutral but about 1% is ions (secondary ions) that can be collected and mass-analyzed. Both positive and negative secondary ions can be analyzed. Identities for these secondary ions are proposed based on their masses and fragmentation patterns. In ToF-SIMS imaging, the analysis area is sectioned into pixels (2D) or voxels (3D) and a full spectrum is collected from each region. An image is then constructed by displaying the intensity of a given peak at each square on a color scale.

Because static secondary ion mass spectrometry (SIMS) probes to a depth less than 2 nm, and because proteins are 5 nm or more in size, SIMS looks at only one "face" of the protein molecule. This selective data set can be used to ascertain information of protein orientation and conformation [12] (Fig. 31.2).

Sum Frequency Generation

Sum frequency generation (SFG) spectroscopy can be used to determine the orientation of adsorbed proteins [13,14]. For SFG vibrational spectroscopy, visible and infrared light beams are overlapped in time and space at a surface or interface. The sum frequency signal is generated at locations without inversion symmetry (e.g., interfaces)

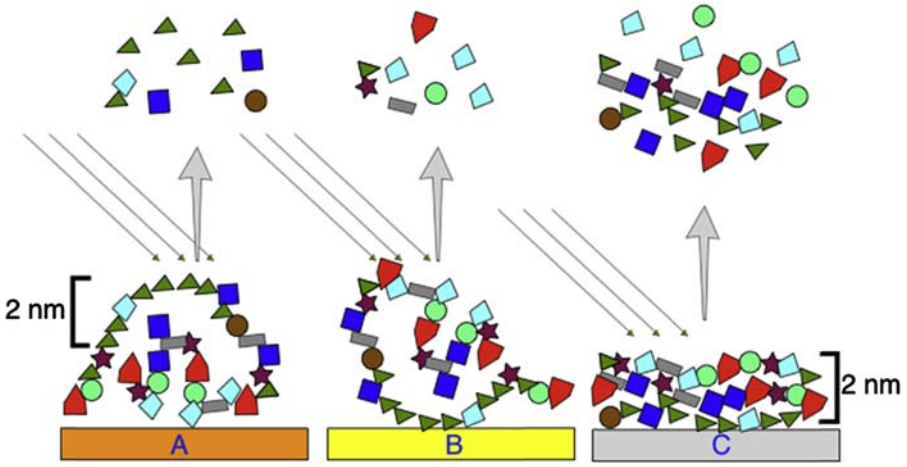

FIGURE 31.2 Graphical description of the mechanism by which static secondary ion mass spectrometry (SIMS) probes protein orientation and conformation. *Thin arrows* represent the SIMS primary ion beam that liberates amino acid fragments from a surface zone less than 2 nm deep. *Larger gray arrows* represent the direction of emission of amino acid fragments. *Colored geometric shapes* are various amino acids (20 different amino acids in most proteins). For surface "A," the *green triangles* are within the 2-nm sampling depth. Because most of the green triangles are in the outermost 2 nm of the protein, there are many green triangles in the emitted amino acid "cloud" (these amino acids are then detected and sorted by the mass analyzer). For a different surface, B, the "green triangle" face of the protein is buried at the solid–protein interface, so few green triangles are seen in the emitted amino acids. For case "C," the protein is highly denatured (flattened to the surface), and thus all of the protein is within the 2-nm sampling depth and all amino acids are seen in the SIMS emission.

owing to nonlinear optical frequency mixing. When the vibrations of interfacial species satisfy the SFG-selection rules (e.g., both Raman and infrared [IR] active), the SFG signal is enhanced. Owing to the nonlinear optical selection rules of SFG, only ordered protein layers at the interface are detected. Disordered proteins, either on the surface or nearby in solution, are not detected. In analogy to Raman or IR spectroscopy, the amide I modes can provide detailed information about the folding and structure of interfacial proteins.

SFG spectra can be calculated and compared with experimental data to aid in the spectral interpretation. For example, couplings between the amide groups can be determined from atom coordinates in protein structure files. Nearest-neighbor couplings between amide groups can be calculated using ab initio methods that give the coupling as a function of the dihedral angle between the neighboring amide moieties. Non–nearest neighbor couplings are calculated with a coulomb-like transition dipole coupling model. Then the IR and Raman modes of the protein can be calculated and used to calculate the vibrational SFG response.

Surface Plasmon Resonance

Surface plasmon resonance (SPR) takes advantage of the excitation and perturbation of surface plasmons (quantized vibrations of mobile electrons within a metal) to probe events occurring at a metal surface (usually gold). If solution-phase molecules adsorb or interact at a surface, this alters these plasmon vibrations and leads to changes in the vibration resonance frequency. We relate this frequency change to the mass and refractive index of the adsorbates. SPR instrumentation is well-suited for studying biomolecule (protein) adsorption and biomolecule interactions at interfaces [15,16].

Quartz Crystal Microbalance With Dissipation Monitoring

Quartz crystal microbalance with dissipation monitoring (QCM-D) can be used to measure changes in mass and viscoelastic properties of adsorbed and immobilized proteins as a function of time [17,18]. QCM-D is based on the piezoelectric effect. When a voltage is applied to a piezoelectric quartz crystal, the material begins to oscillate. The frequency of oscillation will change depending on the amount of material deposited onto the quartz crystal. The dissipation is determined by measuring the signal decay once the voltage to the quartz crystal is turned off (much like the decay in sound of a struck bell). Viscoelasticity, or the rigidity of the deposited layer, will cause the voltage to decay at different speeds. The mass adsorbed (in ng/cm^2) can be calculated using either the Sauerbrey or Voigt model. Analysis used a harmonic overtone (e.g., seventh) for calculating the Sauerbrey mass or multiple

harmonic overtones (e.g., third, fifth, seventh, ninth, and eleventh) for calculating the viscoelastic Voigt mass. The Sauerbrey model assumes an inverse relationship between the change in frequency (Δf) and mass (Δm):

$$\Delta f = -\frac{2f_0^2}{A\sqrt{\rho_q \mu_q}}\Delta m$$

where f_0 is the resonant frequency, A is the quartz crystal area, ρ_q is the density of quartz (2.648 g/cm^3), and μ_q is the shear modulus of quartz for AT-cut crystal (2.947×10^{11} g cm^{-1} s^{-2}). This model can be used only when the dissipation change is low, typically less than 5% of the change in frequency. In this case, the protein is considered rigid. If dissipation occurs, the Sauerbrey model will underestimate the mass. If the dissipation is too large to use the Sauerbrey model, the Voigt model can be used, which models the changes in viscoelastic properties as a damper and spring. A hydrated, flexible protein at a surface might be best modeled using the Voigt model.

Atomic Force Microscopy

Atomic force microscopy (AFM) has been widely used to image the organization, dynamics, and conformations of proteins directly at interfaces [19–23]. AFM generates images by passing a flexible, sharp-pointed stylus over a specimen and converting the x, y, and z position information of the stylus to electrical signals that can then be translated into image pixels representing the height and dimensions of the object being probed. Spatial resolution for contemporary instruments can approach 0.1 nm. From AFM data, one can also infer information on the molecular-scale biomechanics of proteins and their motions on surfaces [24].

Multivariate Methods for Analyzing Surface Molecular Information

Multivariate statistical methods, sometimes referred to as chemometrics, provides powerful mathematical tools to discover trends and correlations in large data sets such as are often obtained with modern surface analysis instrumentation [25–27]. The most widely used of these methods is principal components analysis (PCA), although many other methods are available. The application of multivariate analysis methods to study proteins at interfaces has been reviewed [28,29].

Conformational Stabilization for Biomolecules at Interfaces and Frozen-Hydrated Surface Studies

A challenge in the precise use of proteins at interfaces is to stabilize structures to permit analysis (especially for analytical techniques requiring high vacuum such as XPS and SIMS). Also, in stabilizing delicate conformational structures at interfaces, enhanced bioactivity can be realized. Three techniques have been especially useful for such structural preservation: freezing the proteins, freeze-drying (or critical-point) drying, and surrounding proteins with a stabilizing agent. Frozen-hydrated studies under ultrahigh vacuum for XPS have been described [30]. Key advances in cryogenic transmission electron microscopy [31] will probably lead to improved methods for studying proteins by cryo-XPS and cryo-SIMS. Freeze-drying can be effective for preserving protein structure and interfaces; coupled with preservation agents such as trehalose or sorbitol, excellent preservation can be realized [32,33]. The use of trehalose to preserve protein structure in surface studies was clearly demonstrated in a published study [34].

Iodine 125–Labeled Proteins

Labeling a protein with an I^{125} (gamma emitter) tag, although an older method, has excellent advantages for specificity and sensitivity in studying protein–surface interactions for medical devices or extracellular matrix scaffolds. An extensive review covering the methodology and rationale for I^{125} studies has been published [35].

TECHNIQUES AND TECHNOLOGIES FOR PRECISION IMMOBILIZATION AT SURFACES

This section will review many of the methods that have been used to deliver protein signal from surfaces with precision and specificity (in contrast to simply immobilizing the protein to the surface with little control of orientation or conformation). To anchor proteins to surfaces, we can consider three general classes of interactions:

noncovalent, covalent, and ionic. These are, respectively, weak bonds (although many of them to provide stronger interactions (i.e., cooperativity)), strong bonds (direct chemical attachment through directional chemical bond formation), and electrostatic ("plus–minus" interactions). A variety of potential attachment methods will be described that not only anchor the protein to the surface but permit some control of orientation and conformation to enhance signal delivery to living systems.

Hexahistidine Tags

A tag on a protein molecule consisting of a short chain of six histidine residues (HIS tag) can added precisely at the protein C or N terminus. Because the HIS tag will bind to a nickel–nitrilotriacetic complex (Ni-NTA), and the Ni-NTA complex can be bound to a surface of interest, this binding event can be used to orient the protein (Fig. 31.3). If the active or recognition site is near the C terminus, it would be best to place the HIS tag at the N terminus (and vice versa). There are many published examples of using HIS tags to control protein orientation [36,37].

Ionic Charge and Charge Control of Orientation

With an appreciation that protein molecules have many "faces," each with different molecular characteristics, some faces will be, on average, more positively charged and others will be more negatively charged. It follows that if a biomaterial or scaffold surface is positively charged, it should interact more strongly with a negative domain on the protein surface. This relatively straightforward rationale for orienting protein molecules has been shown in a number of studies to confer much enhanced recognition specificity to bound proteins.

In a study focused on engineering the orientation of surface immunoglobulin G (IgG) proteins via an electrostatic charge, a strong effect of surface charge was noted on the orientation of the protein [38]. This study is schematically illustrated in Fig. 31.4. Positively charged self-assembled monolayers (SAMs) (amino head group) and negatively charged (carboxylic acid head group) SAMs were used as substrates to adsorb and interact with IgG. A bare gold substrate was used as a control. IgG is made up of two Fab subunits and an Fc subunit (Fig. 31.4A). Because of their different amino acid compositions, the Fc subunit is more negatively charged at physiological pH and the Fab subunit is more positively charged. First, the IgG protein was enzymatically cleaved to Fab and Fc fragments. SIMS spectra were taken of Fab units and Fc units to form a "learning set" for subsequent PCA analysis

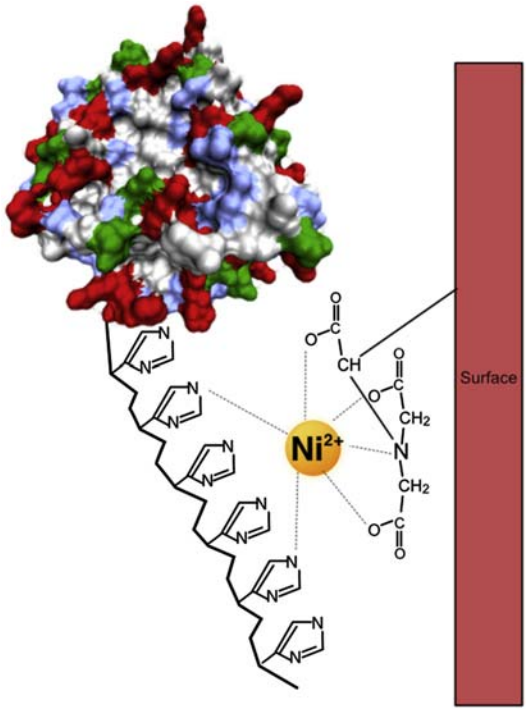

FIGURE 31.3 A nickel–nitrilotriacetic complex can bind to a short histidine chain introduced into a protein. Thus, depending on the specific, controllable site of the histidine chain on the protein, the protein can be oriented to the surface.

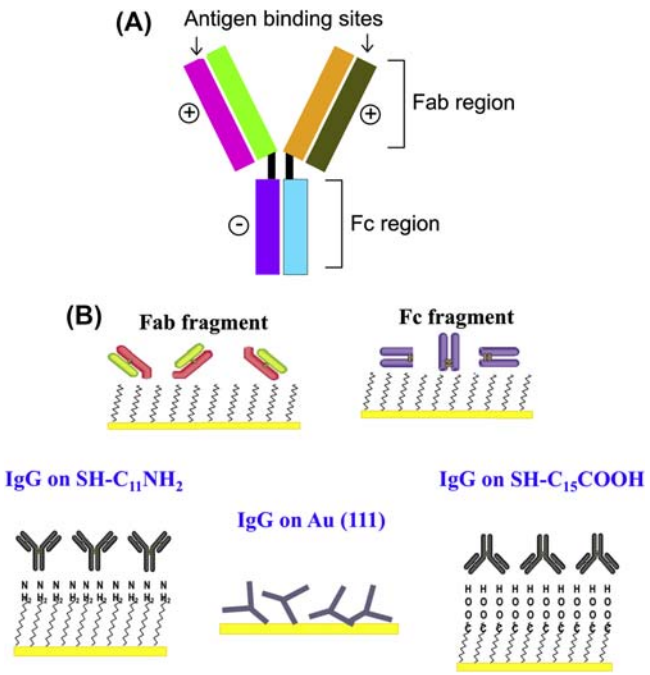

FIGURE 31.4 Schematic representation of a published study on antibody orientation [38]. (A) The subunits of an immunoglobulin G (IgG) molecule with an indication of their relative charge states at physiologic pH. (B) The IgG was dissociated into the Fab and Fc portions for secondary ion mass spectrometry (SIMS) analysis. Then the IgG molecules were adsorbed onto an amino-terminal self-assembled monolayer (SAM) (positively charged surface), a carboxylic acid terminal SAM (negatively charged surface), and a gold surface (control). (B) SIMS analysis was performed on all specimens, demonstrating that the images represent the predominant orientation based on an analysis of secondary ion amino acid fragments expected for the Fc portion of the molecule "up," the Fab portion "up," or random distribution.

(Fig. 31.4B). Then IgG was adsorbed to an amino-terminal (head group) SAM, a carboxylic acid terminal SAM, and gold. SIMS analysis looking into the top 1−2 nm of these films provided mass spectra showing amino acid distributions. For the amine surface, the SIMS amino acid distribution was suggestive of the Fab portion. For the carboxylic acid surface, the SIMS amino acid distribution was compositionally closer to that of the Fc portion. No such separation was seen for the molecules on the gold surface where no charge-directed orientation would be expected. An antigen to the IgG on the amine surface where the Fab portion was facing out to the liquid interface would be expected to have much higher molecular recognition.

Other studies using charged substrates to orient proteins (osteopontin, fibronectin subunit, and $FNIII_{7\text{-}10}$) demonstrated that the surface charge can strongly enhance bioactivity (antibody recognizability and cell attachment) (Fig. 31.5) [39,40]. This effect is presumably induced by orienting the proteins so recognition motifs interacting with cells and other proteins are more accessible to the biological environment.

Collagen to Control Protein Orientation

Although best known as a structural protein, collagen type I binds biospecifically to least 50 other biomolecules [41]. When biomolecules bind biospecifically to collagen I, they are conformationally stabilized in a geometry that is optimized to deliver signals or perform other functions. Collagen I can be exploited to deliver protein signals with precision. It is well-suited for this purpose for a number of reasons: it is inexpensive, well-tolerated in vivo, already approved for many medical applications, and chemically robust, and in its triple helix configuration, it is easily oriented and axially symmetrical, so there is no "up" or "down" when it is immobilized onto a surface. In a few studies, collagen was shown to be remarkably effective in enhancing the bioactivity of molecules bound to it. For example, in one study, collagen I surfaces were treated simply by dipping into osteopontin (OPN) solutions [3]. Such surfaces had much higher attachment of bovine aortic endothelial cells than surfaces onto which the same amount of OPN was covalently immobilized using carbonyl diimidazole chemistry. Collagen I with no OPN was relatively ineffective for cell adhesion. A similar amplification of OPN's ability to mediate cell adhesion when bound to collagen I was seen in a related study [42].

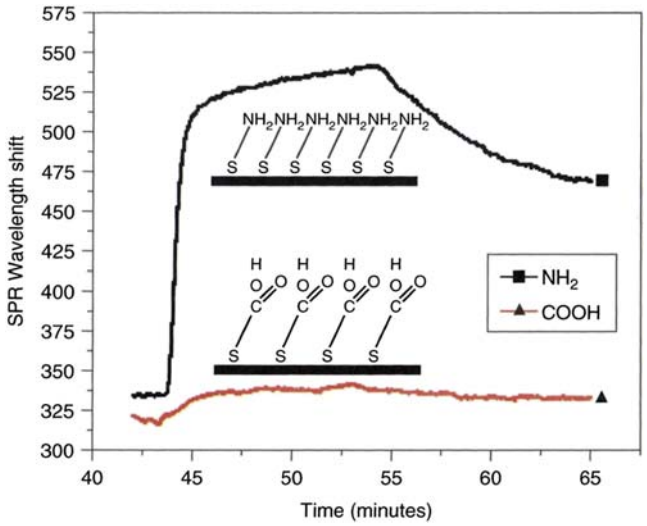

FIGURE 31.5 Surface plasmon resonance (SPR) measurement of the amount of a monoclonal antibody to the cell binding region of peptide $FnIII_{7-10}$ for peptide $FnIII_{7-10}$ on COOH− and NH_2− self-assembled monolayers, each adsorbed with equivalent amounts of $FnIII_{7-10}$. Although all surfaces have the same amount of adsorbed peptide, only peptide on the amine surface is strongly recognized by the antibody.

Immobilization of Proteins in Lipid Layers and Tethered Lipid Bilayers

The lipid bilayers of living cells are evolutionarily optimized system for delivering appropriate protein signals with high bioactivity and specificity. Proteins are conformationally stabilized in the lipid environment and oriented so their recognition elements are optimally available (Fig. 31.6).

In analogy to nature's method of delivering protein signals, using lipid bilayers, synthetic systems have been envisioned using assembled lipid layers to constrain and orient proteins. Fig. 31.7 offers some possibilities. A number of publications have described such strategies for controlling proteins at interfaces in some detail [43–46].

Streptavidin for Biomolecular Orientation Control

Streptavidin protein has a number of advantages as a tool for orienting proteins and delivering signals from proteins. Its symmetrical tetravalency offers many possibilities for surface tethering, protein tethering, and molecular orientation. Streptavidin is robust, it has an exceptionally high biotin binding affinity ($K_a = 10^{14}$), and it is not too large, which reduces steric concerns (53 kDa). Fig. 31.7B illustrates one option for using streptavidin to deliver signals from appropriately oriented proteins. Many publications are available on using streptavidin to immobilize other proteins and control their orientations [47–49].

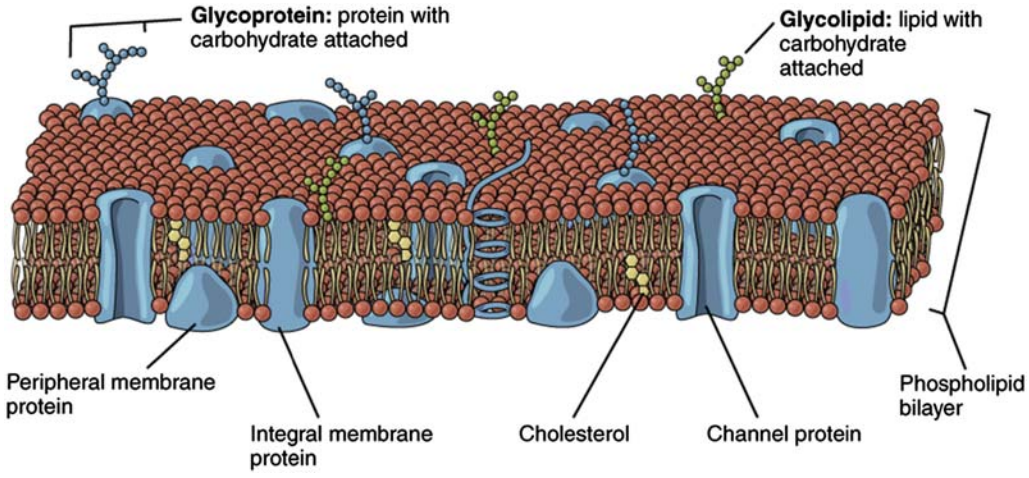

FIGURE 31.6 Proteins that are embedded within a cell's lipid bilayer membrane are conformationally stabilized and optimally oriented to deliver signals. *Figure from Wikimedia Commons.*

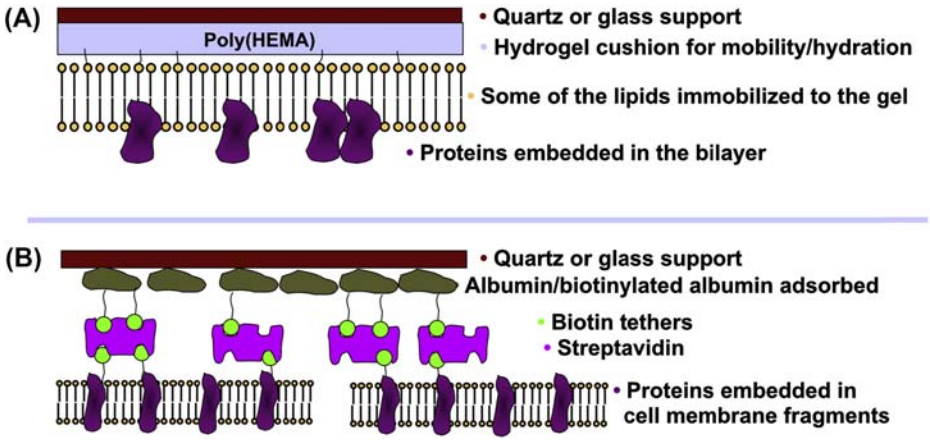

FIGURE 31.7 Delivering protein signals with proteins embedded in lipid bilayer-like structures. *Based on a diagram from H. Vogel.*

Hydroxyapatite for Protein Signal Delivery and Orientation Control

Hydroxyapatite (HA) calcium phosphate mineral [$Ca_{10}(PO_4)_6(OH)_2$] has long been known to interact biospecifically with proteins [50–52]. Indeed, HA–protein interactions are critical for bone formation and bone strength is improved by protein–HA interactions that inhibit the formation of large HA crystals. Thus, HA surfaces represent a unique option to control protein orientation and bioactivity. In one study using XPS and SIMS to compare osteocalcin adsorption with both calcium phosphate and silica surfaces, silica was found to induce the denaturation of the osteocalcin compared with calcium phosphate [52]. The specificity of HA for purifying antibodies has been well-described in the literature [53]. In excellent studies demonstrating the biospecificity of HA–protein binding as analyzed by nuclear magnetic resonance spectroscopy, the investigators determined precisely which portion of a statherin peptide interacts with HA and which is conformationally mobile and free from surface interaction [54,55].

Other Options to Control Proteins at Interphases With Precision

Not all potential methods for protein control at surfaces and interfaces have been elaborated upon in this article. Nevertheless, it is useful to mention a few other possibilities. If antibodies (particularly monoclonal antibodies) can be oriented at a surface, the possibilities of using those surface antibodies to orient and control other proteins in the surface zone become viable options for biosurface construction [48]. Strategies using NeutrAvidin Protein A complexes and protein G (antibody binding proteins) at the interface can also direct antibody orientation [56,57]. Electrical fields might be used to orient antibodies and other proteins at interfaces [58]. An extensive general review on antibody orientation (with particular focus on immunoassays) was published [59]. Finally, surface imprints prepared from protein templates might find application to create oriented, controlled protein surfaces [60–62].

In addition to the techniques for creating ordered protein surfaces, there are a few "platform" technologies that are important and supportive for such precision surfaces. One set of technologies deals with incorporating appropriate functional groups into the surface. These functional groups are the anchors or handles to affix proteins or the molecules that lead to organized protein films. Important functional groups are $-NH_2$ and $-OH$; other chemical groups are $-SH$, $-N_3$ (azide), alkynes, and acrylate double bonds. Glow discharge plasma deposition is a powerful method for surface modification to incorporate anchored functional groups [63,64], although many other methods are available [65].

Another set of platform technologies for precision-controlled protein surfaces uses nonfouling (cell and protein-resistant surfaces) regions on the surface [66]. Recognition elements (i.e., oriented protein molecules) can be spaced in a "sea" of nonbioadhesive chemistry so that the recognition event is not fouled by the nonspecific adsorption of proteins or by cell attachment. Many technologies have been developed to create nonfouling surfaces; the most commonly used ones are poly(ethylene glycol) surfaces [67] and zwitterionic polymer surfaces [68,69].

CONCLUSIONS

There are numerous possibilities for controlling the orientation, conformation, and density of proteins at surfaces with precision and specificity. Such precision control is sharply in contrast to nonspecific adsorption or nonspecific chemical immobilization, the protein–surface technologies that have dominated the field since the 1960s. Precision

engineered protein surfaces exhibit higher "signal-to-noise" (i.e., they are more effective in fulfilling their intended purpose) and are less likely to be interrogated by living systems as denatured proteins to be targeted for destruction. The shift in thinking from simply applying proteins to surfaces and/or scaffolds with little control, to engineering surfaces with biological specificity and control, is facilitated by newer methods for creating such surfaces and the technologies for analyzing these surfaces. This review is written to start the reader on the path to thinking about the merits of the precision control of protein structure and function at synthetic and biological interfaces. There is a huge literature on this subject; thus, starting from this basic tutorial, a sophisticated, nuanced approach to the subject of organized proteins at interfaces can be developed.

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CHAPTER

32

Natural Origin Materials for Bone Tissue Engineering: Properties, Processing, and Performance

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INTRODUCTION

Advances in the medical field have increased life expectancy worldwide and increased age-related pathologies that are responsible for reducing the quality of life for elderly people. Bone injuries may be severe among the aging population and can result in morbidity and significant socioeconomic costs. Thus, there is a need for new and efficient regenerative strategies to optimize patient outcomes.

Current options for treating large bone injuries are unsatisfactory because they rely on the use of autografts, allografts, and scaffolds associated with cells and/or biological factors. Each option has major limitations such as reduced bioactivity, the risk for the transmission of pathogens and inflammation, the need for additional surgery, limited supply, inadequate size and shape, and morbidity associated with the donor site. To address these limits, advanced tissue engineering-based approaches have been pursued to create grafts to repair and regenerate damaged tissues [1]. The engineered scaffolds should be cytocompatible and mimic the native tissue to present biochemical and mechanical cues. In this way, similar biological functions can be promoted, overcoming issues such as donor site scarcity, immune rejection, and the transfer of pathogens.

The bone tissue engineering field has progressed. The main strategies have focused on the combination of cells, biologically active molecules, and a temporary three-dimensional (3D) porous scaffold.

The evaluation of scaffolds for bone tissue engineering is generally composed of cellular interactions as well as an assessment of the scaffold microstructure and mechanical properties. These properties are important for the success of the scaffold for bone tissue engineering applications. The scaffold microstructure (e.g., pore size, shape, and interconnectivity) is an important characteristic for the success of the developed scaffold because it should allow cell migration, nutrient and waste exchange, neovascularization and angiogenesis, and bone ingrowth. Mechanical properties are also a critical parameter for bone remodeling because they should ensure the formation of a new tissue once implanted while maintaining its structural integrity.

Because load-bearing applications require porous structures with improved mechanical performance, we believe that solid porous structures can be more appropriate for connective tissue engineering applications than matrices from hydrogels. Therefore, special attention will be given to processing techniques used to prepare foams and meshes and their performance in vitro and in vivo, alone or combined with cells, in the context of bone tissue engineering. Table 32.1 provides a quick overview of natural-based polymers and ceramics and their properties, processing methods, and performance, which will be discussed in more detail later in the chapter.

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TABLE 32.1 Natural-Based Polymers and Ceramics and Their Properties, Processing Methods, and Performance

	Materials	Properties	Processing Methods	Performance	References
Natural-Based Polymers	Chitosan	Crystallinity dependent on degree of deacetylation Degraded by lysozymes Insoluble in aqueous solutions pH < 7 Similar to glycosaminoglycans (GAGs)	Phase separation Lyophilization Porogen technique Melt-based technique Gas foaming Freeze gelation Particle aggregation Rapid prototyping Nanofiber production	Induce minimal foreign body response Accelerate wound healing Antimicrobial properties Low mechanical properties	[3,5–7,9–11]
	Collagen	Most abundant structural protein Can be isolated from different tissues and species Insoluble fibers High tensile strength and stability Degraded by matrix metalloproteinases Abundant functional groups	Freeze-drying Porogen technique Electrospinning Computer-aided manufacturing Solid free-form fabrication	Low antigenicity Biocompatible High porosity Facility for combining with other materials Low mechanical properties	[19–21,24–27]
	Gellan gum	Similar to GAGs Soluble in water Insoluble in ethanol Mechanical and gelation properties dependent on degree of acetylation Gelation upon interaction with monovalent and divalent cations Processed under mild conditions Easily modified	Casting approaches Freeze-drying Electrospinning Wet spinning Bioprinting	Biodegrades over weeks to months Induces low inflammation Not cytotoxic Low mechanical properties	[33–35,38,39]
	Polyhydroxyalkanoates	High melting points Low glass transition temperatures High crystallinity	Solvent casting Particle leaching Phase separation Freeze-drying Electrospinning	Biodegradable Biocompatible Brittle Slow degradation Induces secretion of cytokines necessary for wound healing Minimal inflammatory response	[46,47,49–51]
	Silk fibroin	Formation of β-sheets	Salt leaching Freeze-drying Lyophilization Inverse opal Electrospinning Three-dimensional (3D) plotting	Biocompatible Biodegradable High mechanical properties Not immunogenic Not cytotoxic	[55–60]

	Starch	Degraded by several enzymes (e.g., amylases)	Solvent casting Particle leaching Freeze-drying Supercritical assisted phase-inversion Wet spinning Water-in-oil emulsification Coprecipitation	Biodegradable Brittle	[68−71,73,74]
Natural-Based Bioceramics	Calcium phosphates	Crystalline Similar to minerals found in bone Low resorption rate	Foam replica Solid free-form 3D printing Gas foaming Electrospinning	Biocompatible Bioactive Osteoconductive Osteoinductive Not toxic Not inflammatory Not immunogenic Promotes osteogenesis and osteointegration Formation of apatite layer	[80,85−89]
	Silicate ceramics	Fine-tuned mechanical properties, bioactivity, and degradation rates	Sol−gel Foaming Particle leaching Melt-derived approach Melt spinning	Osteointegration Brittle Induces bone formation Slow degradation rates Formation of apatite layer	[91−97]

NATURAL-BASED POLYMERS

Natural polymers are widespread in nature. Those polymers are formed during the growth cycles of many organisms and are obtained from renewable sources such as plants, animals, and microorganisms. A large variety of natural polymers are available with potential interest for the production of scaffolds because their characteristics better mimicry tissue natural extracellular matrix (ECM). Natural polymers can be fine-tuned in terms of their degradation rate, biological activity, porosity, charge, and mechanical strength by changing the polymer concentrations or polymerization conditions, or by introducing functional groups. Moreover, their susceptibility to enzymatic or hydrolytic degradation may indicate great susceptibility to being metabolized under physiological conditions.

CHITOSAN

Chitosan is a deacetylated derivative of the biopolymer chitin. Chitin is a natural polysaccharide composed of β(1→4)-linked *N*-acetyl-D-glucosamine residues and is the most abundant polysaccharide in nature after cellulose. Chitin can be found in the exoskeleton of invertebrates such as crustaceans, insects, and spiders, and in the cell walls of most fungi and many algae [2].

Structurally, chitosan is a linear polysaccharide consisting of *N*-glucosamine (deacetylated unit) and *N*-acetyl glucosamine (acetylated unit) units linked by β(1 → 4) glycosidic bonds. The molecular weight may vary between 300 and 1000 kDa, with a degree of deacetylation (glucosamine/*N*-acetyl glucosamine ratio) that usually can vary from 30% to 95%. The degree of crystallinity of chitosan is mainly controlled by the degree of deacetylation that is maximum for both chitin (i.e., 0% deacetylated) and fully deacetylated forms (100% chitosan) and minimum for intermediate degrees of deacetylation [3].

Chitosan is degraded in vivo by means of lysozyme, which hydrolyzes glucosamine−glucosamine, glucosamine-*N*-acetyl-glucosamine, and *N*-acetyl-glucosamine-*N*-acetylglucosamine bonds. The reaction results in oligosaccharides that can be incorporated into glycosaminoglycans (GAGs) or glycoprotein pathways or metabolic pathways or excreted. The degradation rate is inversely related to the percentage of crystallinity that can be controlled mainly by fine-tuning the degree of deacetylation. Highly deacetylated forms (e.g., 85%) exhibit the lowest degradation rates and may last several months in vivo [3]. The degradation products are chitosan oligosaccharides of variable length.

Chitosan is insoluble in aqueous solutions above pH 7, but in dilute acids, the free amino groups are protonated and the molecule becomes fully soluble below pH 5. The pH-dependent solubility of chitosan provides a convenient mechanism for its processing under mild conditions. For example, at pH > 7, chitosan becomes insoluble and precipitates, resulting in hydrogels. Freeze-drying techniques also allow highly porous structures to be obtained by means of freezing a polymer solution (−20°C and −196°C), followed by the removal of solvent through lyophilization. Thus, chitosan can easily be processed into porous scaffolds [4]. If we consider its biological features, as such as an induced minimal foreign body response and accelerated wound healing, and its antimicrobial properties, chitosan is one of the most appealing biomaterials for tissue engineering strategies. In addition, much of the potential of chitosan as a biomaterial for tissue engineering can be partially justified by its structural similarity to GAGs, because it possesses glucosamine residues similar to those of the major components of the cartilage ECM. Because the properties of GAG include many specific interactions with growth factors, receptors, and adhesion proteins, the analogous structure in chitosan may also have related bioactivities. Moreover, the cationic nature of chitosan allows it to interact with anionic GAGs, proteoglycans, and other negatively charged species. This property is great interesting because it may serve as a mechanism for retaining or accumulating these molecules.

Processing Methods

An interesting property of chitosan is that it can be transformed into 3D highly porous structures with a high degree of interconnectivity that allows cells to grow and transport nutrients using various technologies. For example, porous scaffolds can be produced by lyophilizing a frozen solution of chitosan in acetic acid [5]. The freezing process can promote the phase separation of acetic acid from chitosan, which forms ice crystals. Then, acetic acid ice crystals are sublimated during the freeze-drying process, creating pores in the scaffold. The pore size of the obtained scaffolds can vary between 100 and 500 μm, depending on the process parameters, such as the chitosan concentrations, freezing temperature, and thermal gradients. The increase in chitosan concentration results in a decrease in pore size. The main limitation of these structures is that the mechanical properties of pure chitosan scaffolds are low,

which could compromise their use. Jana et al. optimized the solvent acidity and polymer concentration with this processing method [5]. In this way, they were able to produce scaffolds with a higher chitosan concentration (maximum of 12 wt%) than usual (4 wt%), which greatly improved the mechanical properties. In addition, the scaffolds that were produced had better rates of cell proliferation and cell differentiation (Fig. 32.1).

Using a different approach, Costa-Pinto et al. combined the use of porogens (salts, sugars, paraffin, and gelatin) with a melt-based technique [6]. With this approach they were able to increase the level of interconnectivity to 90%, which is adequate for the diffusion of cells into the inner regions of the scaffolds. The pore range obtained was 100–250 μm, which was shown in the literature to be suitable for bone regeneration [6]. An alternative to the use of porogen is a foaming or blowing agent (e.g., sodium bicarbonate) that creates gas bubbles, resulting in the formation of a porous structure [7].

Another method is to use freeze gelation, which is based on exposing a chitosan/acetic acid solution to a sodium hydroxide/ethanol solution at −20°C, inducing phase separation and gelation. Then, acetic acid ice crystals

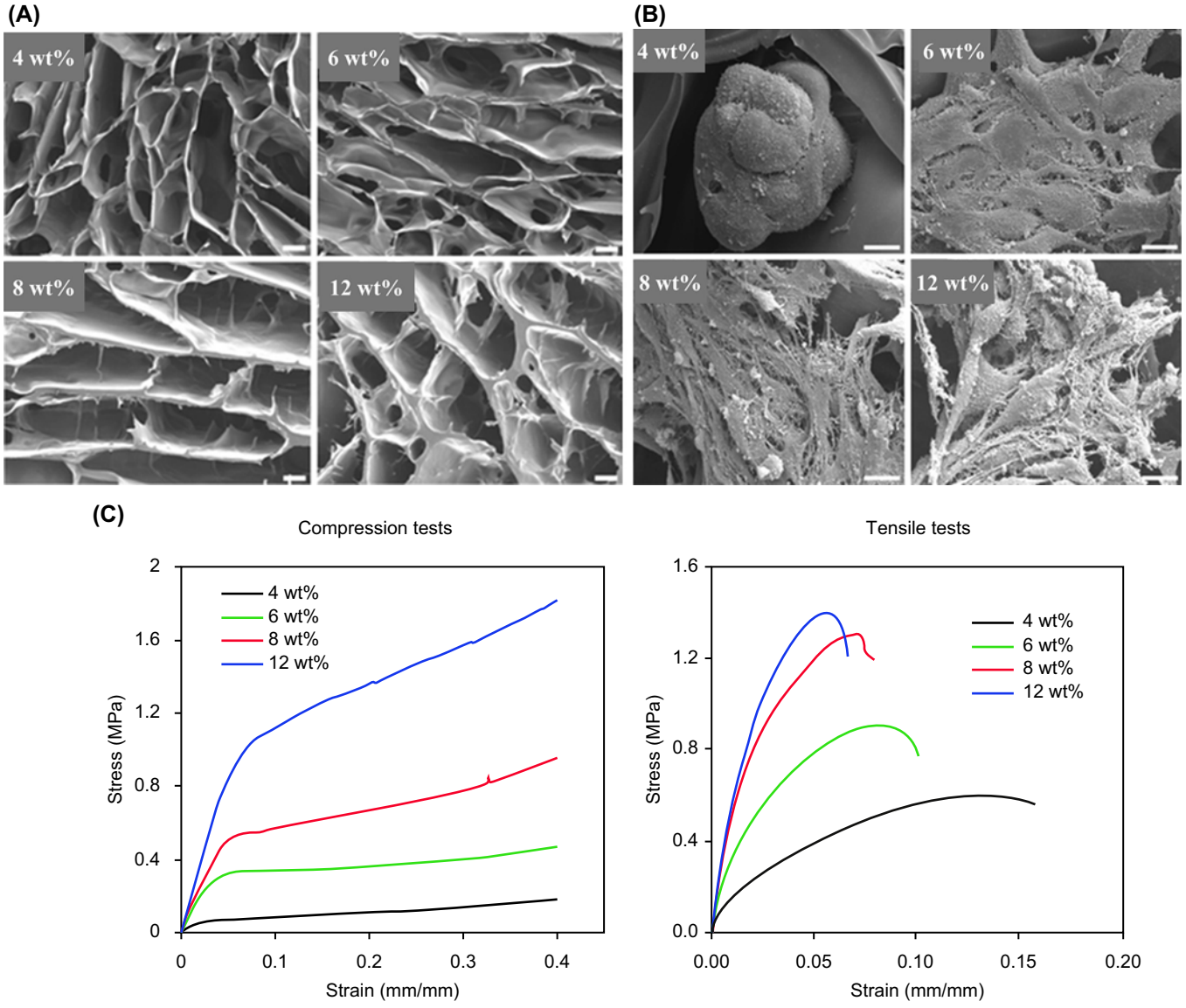

FIGURE 32.1 Scanning electron micrographs of (A) the structure of chitosan scaffolds prepared at different concentrations (scale bars = 100 μm), (B) the cell morphology observed when cultured in chitosan scaffolds at different concentrations (scale bars = 10 μm), and (C) the mechanical properties of chitosan scaffolds prepared at different concentrations. *Adapted with permission Jana S, Florczyk SJ, Leung M, Zhang M. High-strength pristine porous chitosan scaffolds for tissue engineering. J Mater Chem 2012; 22(13):6291–9. Copyright © 2016.*

are formed and the sodium hydroxide/ethanol solution is allowed to air-dry at room temperature instead of lyophilize [8].

Finally, with the intention of developing porous scaffolds, microparticles or microspheres of chitosan can be fused. For example, Malafaya et al. produced a chitosan particle-aggregated scaffold to release biomolecules [9]. With this method, it was possible to obtain a scaffold able to support cell attachment and differentiation and an adequate release profile.

In addition, chitosan scaffolds can be obtained by fiber bonding or, in a more controlled manner, using rapid prototyping technology. The first technology allows the production of a defined structure using computer design and manufacturing [10]. The second allows the production of structures similar to proteoglycans and fibrous proteins found in native ECM [11].

Chitosan in Bone Tissue Engineering Applications

Chitosan-based scaffolds have been designed to imitate some bone properties, which allows the adhesion and proliferation of osteoblasts to be improved. For example, chitosan scaffolds with bioactive ceramics can confer osteoconductive and even osteoinductive properties to the final structure that will guide bone formation. Moreover, they can improve mechanical characteristics. The inclusion of bioactive glass in chitosan scaffolds has been shown to improve scaffold stiffness and promote the deposition of an apatite layer, which is evidence of osteoconductive potential [12]. In addition, Mota Yu et al. showed an increase in cell proliferation and cell matrix mineralization compared with pure chitosan [12].

Calcium phosphate ceramics such as hydroxyapatite (HAp) and β-tricalcium phosphate (β-TCP) were also studied because they mimic bone composition. Maji Dasgupta et al. showed that HAp-chitosan−gelatin nanocomposite scaffolds resulted in mesenchymal stem cell (MSC) adhesion, proliferation, and differentiation [13]. Shavandi et al. studied the addition of HAp and β-TCP to chitosan and observed an increase in mechanical properties that indicated the potential of these scaffolds for bone tissue applications [14].

Calcium phosphate/chitosan biomaterials have been shown to be osteoinductive as well as to have the capacity to promote the formation of new blood vessels, which makes them attractive for bone tissue applications [15]. An in vivo evaluation of calcium phosphate/chitosan biomaterials implanted into rat calvaria defects showed the remarkably accelerated formation and mineralization of new woven bone and histological evidence of primary osteon formation [15].

Using a different approach, chitosan was mixed with a polyester, poly(butylene succinate). The biological assays showed that the scaffolds with amounts of chitosan had higher cell viability, adhesion, proliferation, and osteogenic differentiation [6]. In addition, in vivo tests showed that the scaffolds maintained structural integrity and displayed mild inflammatory response and good integration with the surrounding tissues, including the presence of new blood vessels.

The performance of tissue engineering constructs can be greatly enhanced by incorporating bioactive agents. Chitosan scaffolds with HAp were modified with the peptide Arg-Gly-Asp; its cellular affinity was higher compared with the HAp−chitosan scaffolds [16]. Furthermore, the developed scaffold demonstrated superior osteointegration and bone defect repair and possessed biomechanical properties comparable to those of normal bone.

In a different study, HAp microspheres with chitosan were loaded with recombination human bone morphogenetic protein-2 (rhBMP-2) and the bone-regenerative potential was evaluated in a rabbit radius defect model [17]. The results showed higher rates of new bone formation with rhBMP-2−loaded scaffolds than with rhBMP-2 scaffolds without HAp.

Chitosan has been exploited to increase the mechanical and biological properties of polymers of marine origin, as such as collagen, to provide an alternative to collagen derived from mammalian sources [18]. Overall, scaffolds of collagen extracted from salmon skin had improved mechanical properties and MSC proliferation owing to the addition of chitosan.

COLLAGEN

Collagen is the most abundant structural protein of the extracellular space in mammals. It constitutes around 30% of the total protein weight among which 90% is collagen type I [19].

Collagen has a characteristic triple helix that consists of two polypeptide chains (α1 chains) and a third chain that can differ in its chemical composition (α2 chains). Each chain is composed of approximately 1000 amino acids with a molecular weight of approximately 100 kDa. The amino acid sequence of this molecule is glycine-proline-X and glycine-X-hydroxyproline, in which X is any amino acid besides proline and hydroxyproline. A high proportion of tripeptide sequences found are composed of glycine-proline-hydroxyproline.

Each α chain can form a left-handed helix and the combination of the three chains forms a right-handed triple superhelix structure in which the glycine residues are within the core and the X and Y residues are located in the outer positions [19]. Among the 29 types of collagen, types I, II, III, IV, and V are the most common. Collagen type I is found in almost all vertebrae connective tissues, but especially in tendon and bone. In addition, this collagen has the capacity to form insoluble fibers, which provides high tensile strength and stability to the tissues.

Decades ago, researchers discovered that collagen type I could be isolated from different tissue and different animal species using a simple acid extraction protocol followed by neutralization [20]. Nevertheless, it is most frequently extracted from bovine, which may entail complications as such as the transmission of bovine spongiform encephalopathy [19]. To overcome this limitation, alternative sources have been investigated. For example, Raftery et al. studied collagen derived from marine sources such as salmon skin to work as a platform to promote tissue repair in orthopedics [18].

Human recombinant collagen has also been used for tissue engineering applications. It is a reliable, predictable, and chemically defined source that is free of animal components. Collagen possesses several advantages including abundance, low antigenicity, biocompatibility, and the facility to be combined with other materials. In the human body, collagen is degraded largely through the activity of matrix metalloproteinases [19]. By fine-tuning some features such as the degree of cross-linking, it is possible to modify the mechanical properties and degradation rate of collagen. Furthermore, the abundance of functional groups along its polypeptide backbone makes it highly receptive to binding with genes, growth factors, and other biological molecules.

Processing Methods

Collagen has been reported as a biomaterial to fabricate scaffolds for different tissue engineering applications including bone. Because of its abundancy, it is easy to envision collagen as a platform for tissue engineering and regeneration. One of the most highly used processes to produce collagen scaffolds is freeze-drying [21], in which collagen suspensions are prepared in acetic acid and collagen slurries are allowed to form before the freeze-dry process. The porosity of the obtained scaffolds varies with the conditions chosen for the process. Pawelec et al. showed that the freezing temperature and the addition of a thermal hold on the freezing cycle affect the freezing behavior of the collagen slurry and thus the final pore size [22]. The mechanical properties of the scaffolds that are produced using this technique are determined by the concentration of collagen that is chosen. Nevertheless, scaffolds of pure collagen have poor mechanical properties. To overcome this limitation, physical or chemical procedures (i.e., ultraviolet irradiation, γ-radiation, covalent amine/imine linkage, genipin) are essential to promote the intermolecular cross-linking of collagen [23].

Liu et al. reported an improved approach, i.e., they mineralized the collagen fibrils as a mimetic approach to bone regeneration and promoted intermolecular cross-linking and its superior mechanical properties [23]. This method consists of a synchronized precipitation in which the collagen self-assembles into fibrils and the amorphous calcium phosphate simultaneously transforms into crystalline apatite. The obtained mineralized fibrils are submitted to freeze-drying and then the intermolecular cross-linking of collagen is promoted using carbodiimide chemistry. Finally, the obtained scaffolds are lyophilized again, creating a porous scaffold.

In a different study, ice particulates with different sizes were used as a porogen material to produce scaffolds with different pore diameters [24]. For that, collagen was mixed with the ice particulates and freeze-dried, creating spherical pore structures of different pore sizes and good interconnectivity.

Electrospinning can also be used to produce scaffolds with nano to micron-scale diameter fibers that mimic the fiber's diameters observed in the ECM. Jha et al. concluded that the obtained scaffold did not necessary fully recapitulate the structure of the native fibril to generate a biologically relevant tissue engineering scaffold [25].

Computer-aided manufacturing appears to be a technique that allows the production of highly organized scaffolds. It produces highly porous patterned scaffolds using pure collagen, in which the pore size, shape, and spacing can be controlled [26]. By this method, it is possible to modulate the mechanical properties by changing the number of layers and the shape of the final structure or scaffold.

SeungHyun et al. applied a different approach to obtain a three-dimensional scaffold [27]. They combined a cryogenic plotting system, freeze-drying, and electrospinning to produce a stable scaffold with a controlled pore size. This scaffold had improved mechanical properties, cellular attachment, and a proliferation of MSCs.

Collagen in Bone Tissue Engineering Applications

Type I collagen is secreted by osteoblasts during the process of ossification; it has important roles during mineralization. Therefore, enhanced biological integration with the surrounding tissue may be foreseen when collagen scaffolds are used in vivo. Type I collagen is the basis of several commercial products including Orthopedic Osteon, Orthocell CelGro, Collapat II, Symbios Biphasic Bone Graft Material, Geistlich Bio-Gide, OssiPatch Collagen Bone Healing Protective Sheet, Collatene Fibrillar Collagen Dental Dressing, Collagraft, Infuse Bone Graft, and OssiMend.

Collagen-based scaffolds show great promise for bone tissue engineering applications (Fig. 32.2). In vitro, it was shown that collagen/cellulose scaffolds support cellular adhesion and the phenotype maintenance of cultured human osteoblasts [28]. High levels of osteogenic enzyme, alkaline phosphatase (ALP), and mineral deposition were observed. In vivo, collagen scaffolds implanted in critical bone defects in rat calvaria were biocompatible and highly osteogenic [29]. In addition, the rate of biodegradation was compatible with bone neoformation and vessel neoformation.

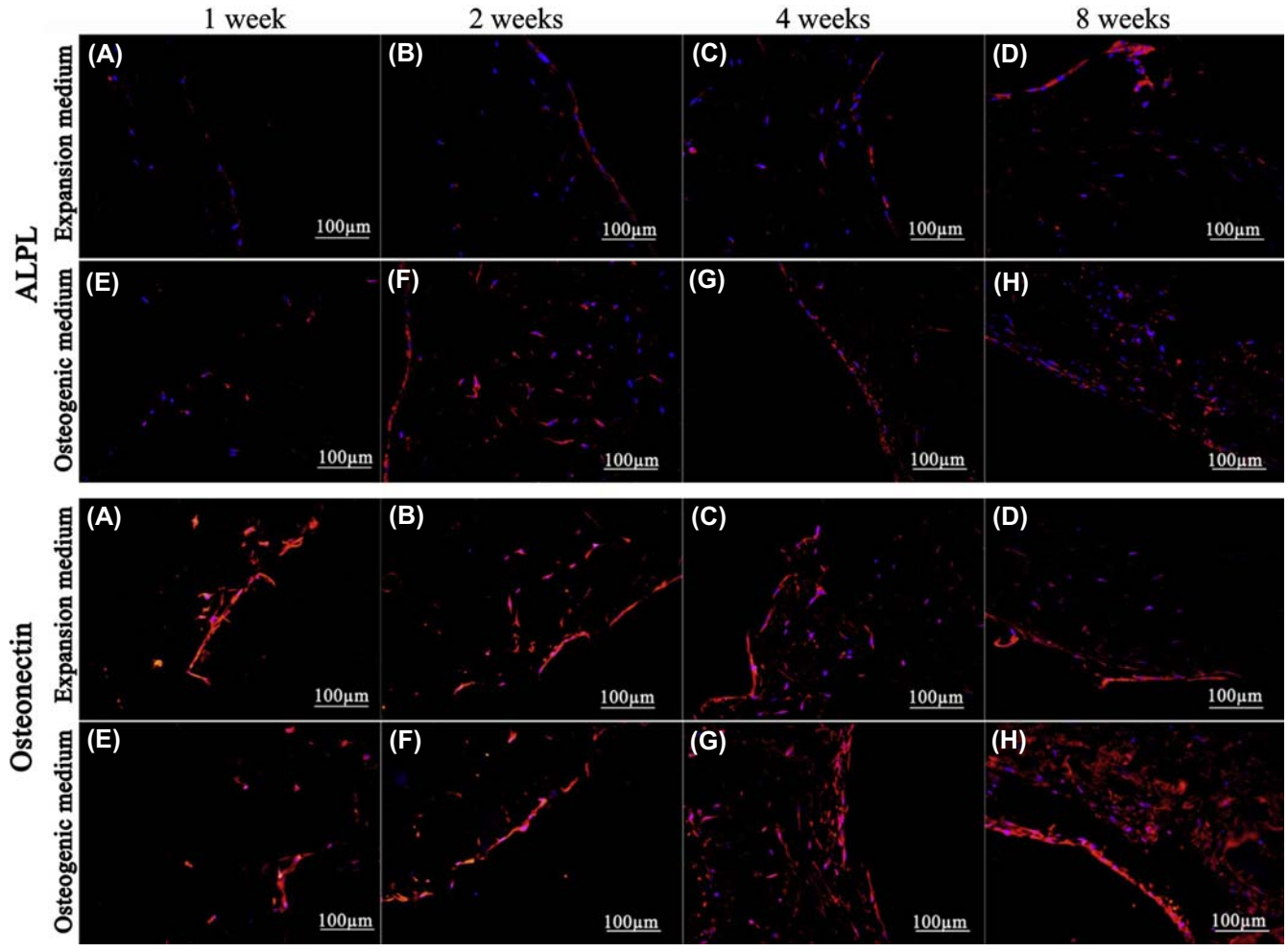

FIGURE 32.2 Immunohistochemical analysis of osteogenic markers (ALP and osteonectin) expressed by adipose-derived stem cells cultured on collagen–hydroxyapatite scaffolds: (A–D) in expansion or (E–H) in osteogenic medium, at different time points (1, 2, 4, and 8 weeks) (Magnification ×20). *ALP*, alkaline phosphatase. *Adapted with permission Calabrese G, Giuffrida R, Fabbi C, Figallo E, Lo Furno D, Gulino R et al. Collagen-hydroxyapatite scaffolds induce human adipose derived stem cells osteogenic differentiation in vitro. PLoS One 2016;11(3):e0151181. Copyright © 2016.*

In a different study, with the objective of mimicking the microstructure and composition of bone ECM, scaffolds were produced based on collagen combined with HAp [30]. It was shown that adipose-derived stem cells (ASCs) cultured in collagen−HAp scaffolds differentiated into mature osteoblasts even in the absence of inducing factors (Fig. 32.2). The same strategy was followed by Kon et al., who implanted collagen−HAp scaffolds to treat osteochondral defects of the knee [31]. In a 2-year follow-up report, it was observed that the approach favored osteochondral tissue regeneration in treating knees affected by damage of the articular surface compared with degenerative lesions. These observations represent significant clinical improvement.

GELLAN GUM

Gellan gum is a linear extracellular polysaccharide produced by the bacteria *Pseudomonas elodea*. Structurally, this high−molecular weight gum (≈500 kDa) contains a repeating unit composed of L-rhamnose, D-glucuronic acid, and two D-glucose subunits. The presence of glucuronic acid residues makes this polysaccharide structural similar to native glycosaminoglycans and supports its use for tissue engineering strategies. Gellan gum is soluble in water; it forms a viscous solution and is insoluble in ethanol, forming precipitates. Its ability to form precipitates enable its extraction from bacteria inoculates in a medium with glucose, nitrogen, and inorganic salts [32]. When submitted to a high temperature, it undergoes a reversible transition conformation from a coil to a double helix upon cooling. The temperature necessary for the transition depends on the polymer concentration, the degree of acetylation, and the presence of cations [32].

Gellan gum may be found as high-acyl gellan gum, which is the native acetylated form, and low-acyl gellan gum, which is the commercially available deacetylated form. The difference between these two forms resides in the presence of glycerate and acetate functionalities in high-acyl gellan gum, which in low-acyl gellan gum are reduced to hydroxyl residues. The reduction results from the hot alkaline conditions employed during extraction. The degree of acetylation can greatly influence the mechanical properties and gelation properties of gellan gum. In this sense, high-acyl gellan gum produces soft, elastic, thermoreversible gels after cooling from 65°C, whereas low-acyl gellan gum produces stiff, brittle, thermostable gels after cooling from 40°C. In terms of gelation, divalent cations (e.g., Ca^{2+} and Mg^{2+}) and monovalent cations (e.g., Na^{+} and K^{+}) can interact with polymer backbone. In the first case, gelation occurs owing to ionic cross-linking between the divalent cations and the carboxylate groups, and because of the screening effect, forming stable and strong bonds. In the second case, gelation occurs only as a result of the screening effect, resulting in less stable structures.

Gellan gum matrices have been mostly produced by casting; they biodegrade over weeks to months, which is an attractive characteristic for tissue engineering approaches [33]. However, to understand their degradation pathways and kinetics fully, more studies must be done. Furthermore, they were found to induce low inflammation in vivo [34]. If we consider their biological features, such as being processed under mild conditions and not being cytotoxic, and also their apparent influence during osteogenesis, one can envisage their potential as a biomaterial. Furthermore, because of the hydroxyl groups formed during deacetylation and the free carboxyl group in the glucuronic acid subunit, they can be modified to improve physicochemical and biological properties.

Processing Methods

Gellan gum has emerged as a biomaterial for tissue engineering because of its biological features but also owing to the possibility of its easily being modified on demand. One of the most well-used methods for developing gellan gum scaffolds is casting followed by freeze-drying [35]. For that, a solution of gellan gum is casted into molds, allowed to form a solid gel upon reaction with divalent cations, and then frozen and lyophilized. However, a limitation of these gellan gum structures that is in common with other biopolymers is their low mechanical properties. The scaffolds' pore size obtained using that technique can vary with the size of ice crystals formed during the freezing process. A rapid freezing process results in smaller ice crystals and consequently smaller pore sizes. Other parameters such as the cross-linking solution, stabilization time, and freezing temperature and time may influence the pore size, as demonstrated by da Silva et al. [36]. They showed that a higher degree of slowdown in the cross-linking formation of ice crystals led to the creation of larger pores. In terms of the time of stabilization, it was observed that an increase in stabilization time resulted in smaller crystals and a smaller pore size. Finally, in terms of the freezing temperature and time, sponge-like scaffolds frozen for longer periods of time and higher temperatures resulted in larger pores. The gellan gum sponge-like scaffolds produced by this method had pores ranging

from 100 to 500 μm in diameter. Interestingly, the researchers optimized the parameters, and using this technique, they produced sponge-like scaffolds with thicker wall pores. Pore wall thickening resulted from microstructure reorganization, which increased scaffold flexibility [36].

The structure of the obtained scaffold also depends on the drying process chosen, as shown by Yang et al. [37]. Oven-dried scaffolds had a crack in the surface of the structure, whereas vacuum-dried scaffolds had a relatively smooth surface, and freeze-dried scaffolds had a looser structure. The different methods also influenced the swelling rates and protein release (Fig. 32.3). In addition, the freeze-dried scaffolds had a sponge-like structure and possessed a high swelling rate, as confirmed by Cerqueira et al. [36]. Moreover, the integration of endothelial cells into these promising scaffolds maximized the neovessels' formation and integration of the transplanted cells into the new vasculature.

A different approach used electrospinning to produce ultrafine nanofibers, as performed by Vashisth et al. [38]. They produced blended fibers of gellan gum with polyvinyl alcohol (PVA) about 40 nm of diameter, because gellan gum alone did not form a fiber structure using electrospinning. The less viscous gellan gum–PVA solutions resulted in fibers with a higher diameter. In addition, the use of polymeric blends (e.g., gellan gum with chitosan) enhanced the mechanical properties [33]. To create fibers using gellan gum alone, wet-spinning must be used, as described by Oliveira et al. [35]. In this approach, they extruded a solution of gellan gum through a needle into a solution of ascorbic acid. Afterward, the fibers were allowed to dry at 37°C.

Bioprinting is a different technique that can also be applied to produce custom-made gellan gum–based scaffolds. This technique combines gellan gum with other polymers, producing mechanically stable and robust 3D scaffolds in a core–shell fashion [39].

Gellan Gum in Bone Tissue Engineering Applications

Gellan gum has been proposed as a novel biomaterial for regenerative medical applications. However, its low mechanical characteristics have prompt the need to mix gellan gum with other materials. For example, Gantar et al. used bioactive glass to reinforce gellan gum–composite, sponge-like scaffolds [40]. Even so, the improvements in mechanical properties did not reach values necessary to accommodate biomechanical loading. Nevertheless, human ACSs were able to adhere and spread within the scaffolds. Altogether, this improved scaffold has great potential for bone tissue strategies. A similar approach was pursued with bioactive glass reinforcement but with different amounts of calcium [41]. The formation of apatite was observed only in the presence of calcium. In addition, bioactive glass with higher amounts of calcium had a lower compressive modulus and higher antibacterial properties.

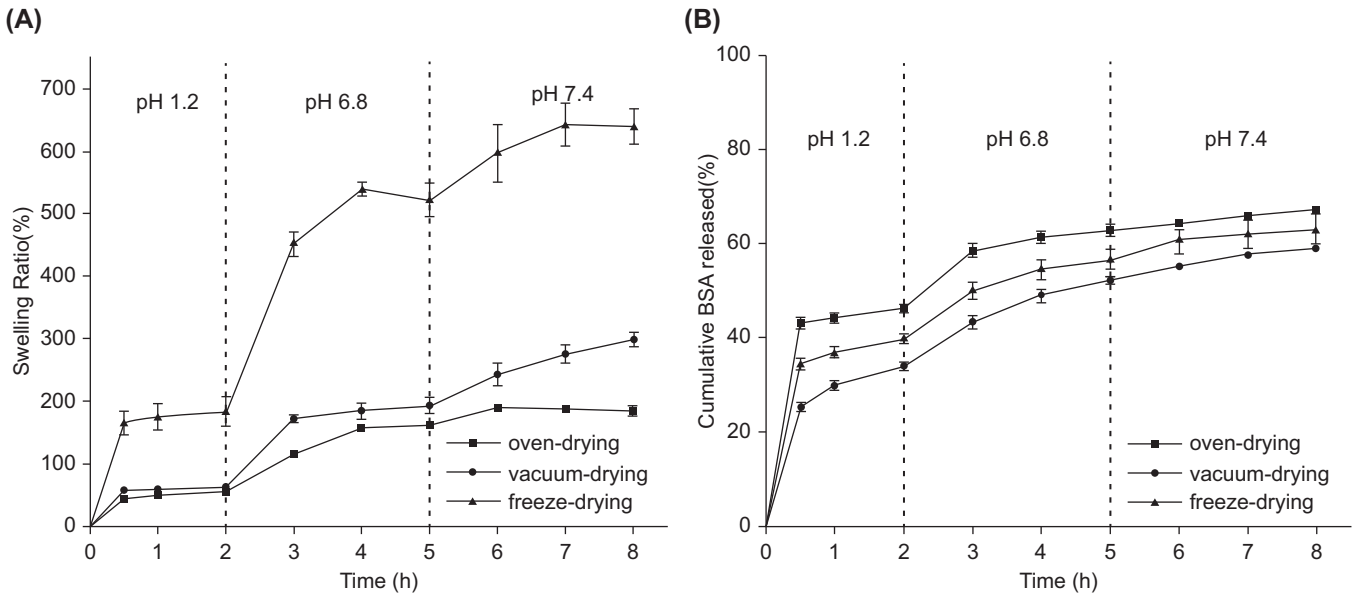

FIGURE 32.3 Effect of different drying processes on: (A) the swelling ratio and (B) protein release. *BSA*, bovine serum albumin. *Adapted with permission Yang F, Xia S, Tan C, Zhang X. Preparation and evaluation of chitosan-calcium-gellan gum beads for controlled release of protein. Eur Food Res Technol 2013;237(4):467–79.*

In a different strategy, Douglas et al. modified the hydroxyl groups present on the gellan gum backbone to improve its suitability for tissue engineering [42]. They incorporated alkaline phosphatase enzyme and observed the induction of an apatite layer and an increase in the scaffold's stiffness. Ultimately, in vitro studies have shown that the apatite layer also enhanced the attachment and viability of the cells. With the intent of improving these developed enzymatically mineralized scaffolds with antibacterial activity, the authors studied the incorporation of zinc [43]. Zinc caused a reduction in the size of the mineral deposits that formed; they had antibacterial activity and promoted the adhesion of bone-forming cells. The same authors compared the use of gellan gum scaffolds modified with alkaline phosphatase or with an extract of seaweed rich in phlorotannins to induce mineralization [44]. The extract of seaweed successfully enhanced mineralization and increased the compressive modulus of the scaffolds. However, when both were present, some mineral formation was observed.

Wen et al. use the fact that gellan gum is biodegradable and its potential as a substrate for apatite deposition to develop a scaffold to repair tissue [45]. For that, they mixed α-tricalcium phosphate and gellan gum, which resulted into a scaffold with increased compressive strength. In addition, the dissolving rate of α-tricalcium phosphate was controlled by adjusting the concentration of gellan gum.

POLYHYDROXYALKANOATES

Polyhydroxyalkanoates (PHAs) are produced by approximately 300 different bacteria upon environmental stress conditions; they function as energy storage. The molecular weight of the PHAs produced is in the range of 200−3000 kDa. PHAs are composed of hydroxy acid monomers connected by an ester bond creating linear polyesters [46]. Depending on the number of carbon atoms present in the monomers, they can be classified as short−chain length PHAs (three to five carbon atoms) or medium−chain length PHAs (6−14 carbon atoms). In addition, different bacteria and substrates result in more than 150 different type of polymers, such as poly 3-hydroxybutyrate (PHB), polyhydroxyvalerate, polyhydroxyhexanoate, polyhydroxyoctanoate, and their copolymers [46]. The PHA family is degraded in nature by a variety of microorganisms such as nonspecific lipases and esterases into carbon dioxide and water. Thus, they are considered biodegradable and biocompatible, which has prompted their use in the medical industry.

PHB, poly 3-hydroxyvalerate (PHBV) and poly 3-hydroxybutyrate-*co*-3-hydroxyhexanoate (PHBHHx) are the most common types of PHAs. Among these, PHB is the simplest occurring form and the first to be discovered. Moreover, it possesses a high melting point (173−180°C) and low glass transition temperature (5−9°C), which results in high crystallinity [46]. Because of this feature, PHB is relatively stiff and brittle and has a slow degradation rate, which is not appealing for most tissue engineering approaches. To overcome this limitation, many studies have used PHBV or PHBHHx [47]. Those have lower melting points and consequently lower crystallinity. In this sense, they are more flexible and undergo degradation at a much faster rate.

Processing Methods

Solvent evaporation is commonly used to prepare PHA scaffolds. For that, the polyester is dissolved in chloroform, allowed to evaporate, and vacuum dried. Nevertheless, other approaches have been studied as an alternative to chloroform for solvents. For example, Anbukarasu et al. used acetic acid to produce films with properties similar to those of films produced using chloroform (Fig. 32.4) [48]. The prepared PHB films possessed crystallinity ranging from 64% at 80°C to 78% at 160°C, whereas, the crystallinity of PHB processed with chloroform was approximately 60.5%. In a different approach, Nemati et al. produced scaffolds by compression molding and particle leaching without organic solvents [49]. For that, they heated a mixture of PHB with HAp and sodium chloride particles up to the melting temperature of PHB, compressed it by uniaxial pressing, and leached out the particles. The scaffolds showed improved mechanical properties in the range of the mechanical properties of cancellous bone and had an interconnected porous structure. The porosity of the scaffolds ranged from 77.2% to 77.5% with pores sizes from 150 to 300 μm.

Porous scaffolds were obtained using sucrose lumps that were soaked in a solution of PHB in chloroform (50). After drying, the sucrose was removed, producing scaffolds with 87.3% porosity and pore sizes of about 120 μm.

PHA scaffolds can also be produced by phase separation and freeze-drying, as was done by Ribeiro-Samy et al. [51]. The polymer was dissolved in organic solvents, and then acetic acid was added to produce two immiscible

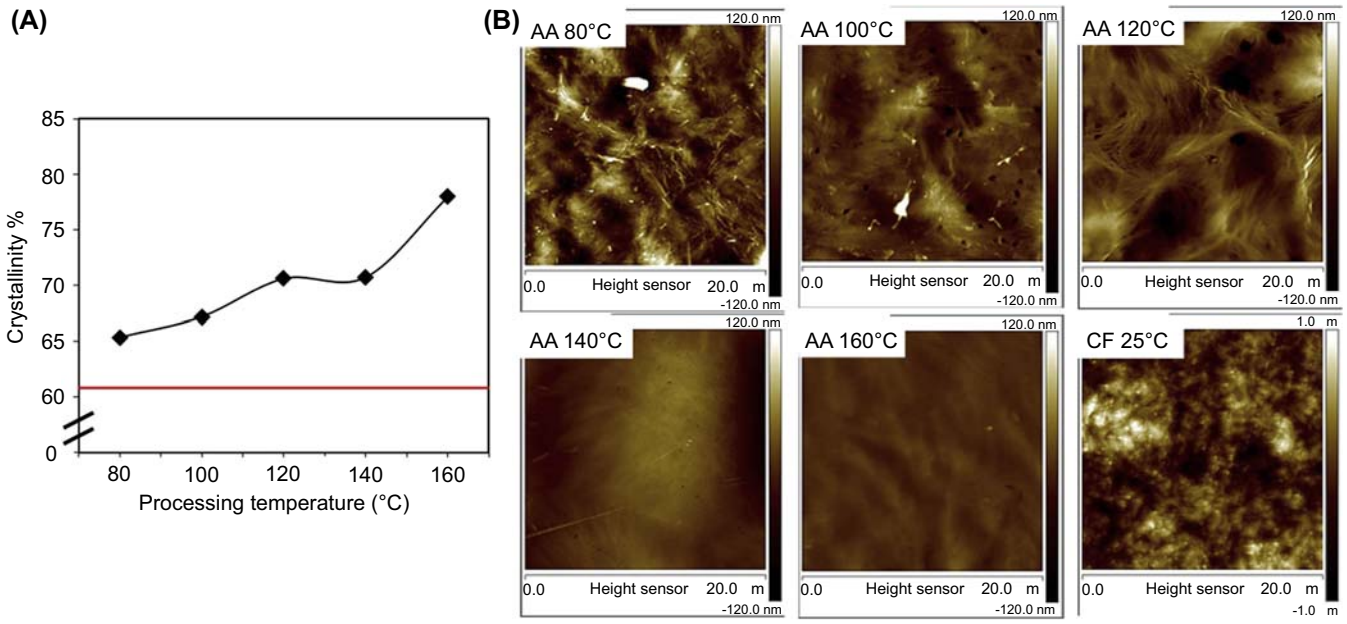

FIGURE 32.4 Properties of polyhydroxybutyrate (PHB) films produced using chloroform(CF) or acetic acid (AA): (A) Crystallinity of films produced using AA (*black line*) or CF (*red line*) with respect to the processing temperature, (B) atomic force microscopy scans of PHB samples processed at different temperatures in AA or CF. *Adapted with permission Anbukarasu P, Sauvageau D, Elias A. Tuning the properties of polyhydroxybutyrate films using acetic acid via solvent casting. Sci Rep 2015;5:17884.*

phases. Afterward, the mixture was frozen and lyophilized. In that way, they obtained scaffolds with a pore size of approximately 164 μm and a porosity of 88%.

Electrospinning is a different technique to produce polyhydroxybutyrate-hydroxyvalerate (PHB-HV) scaffolds [47]. For that, PHB-HV was dissolved in a solution of chloroform with dimethylformamide and a fiber mesh was electrospun. The fibers exhibited diameters ranging from 300 nm to 1.3 μm, similar to the diameters of collagen fibers present in the natural ECM. The developed fiber mesh supported endothelial differentiation, which showed its potential for improving the vascularization in engineered bone tissue.

Polyhydroxyalkanoates in Bone Tissue Engineering Applications

It has been shown that PHBV produced by a metabolic reaction-based model induced the secretion of cytokines necessary for wound healing. PHB scaffolds can easily be fine-tuned with an alkaline solution to increase the proliferation of human osteoblasts and inhibit the growth of bacteria [52]. These features reinforce its potential for tissue engineering applications.

In vitro, the incorporation of HAp into PHB increased biocompatibility and the ability to support the proliferation of human MSCs [53]. In addition, the scaffolds had a higher porosity and improved mechanical properties compared with scaffolds with just PHB.

Cylindrical pins of PHB were produced to evaluate the response of bone upon implantation in the femora of growing rats [54]. For that, the authors modified it with zirconium dioxide to increase radiological contrast values, and with Herafill to increase degradation. Compared with the clinically used poly(lactic-*co*-glycolic acid), poly(glycolic acid), and poly(lactic acid) materials, PHB composites had the advantage of not altering the pH upon degradation, but no degradation was observed after 36 weeks of implantation in vivo. Nevertheless, the composites with Herafill were the most attractive for bone cells and the zirconium dioxide positively influenced the radiological contrast. Nonetheless, it will be necessary to improve the mechanical properties to obtain an appropriate load-bearing material.

Porous 3D implants of PHB seeded with MSCs showed new bone tissue formation in bone defects [50]. It gradually biodegraded, allowing bone tissue ingrowth with a minimal inflammatory response; after 120 days, the defect completely closed. Moreover, the MSCs retained their viability and rate of proliferation, enabling a more effective regeneration.

SILK FIBROIN

Silk is composed of fibroin fibers enfolded by a glue-like coating of sericin. It is produced by several arthropods (e.g., silkworms and spiders). The most common and extensively used type of silk in the textile industry is produced by the silkworm *Bombyx mori*. The composition and structure of silk can vary with the species that produces it, which can influence its mechanical properties, bioactivity, and degradation behavior [55]. It has attracted attention because of its potential use in biomedical applications owing to its biocompatibility and biodegradability [55].

Silk fibroin can easily be separated from the sericin (degumming) using a boiling alkaline or surfactant solution. The result fibers are around 10−25 μm in diameter and are composed of a light chain (≈25 kDa) that is hydrophilic, and a heavy chain (≈350 kDa) that is hydrophobic. The chains are connected by a disulfide bond and are assembled with glycoprotein P25 (≈25 kDa). The heavy chain is composed of glycine-X (in which X is Ala, Ser, Thr, or Val) repeats, which form antiparallel β-sheets, usually called silk-II. The light chains are amorphous blocks in a random coil, which gives elasticity to silk, usually called silk-I [55]. The proportions of silk-I and silk-II may define the final properties of silk. For example, by increasing the amount of silk-II (β-sheet structures), the time of degradation increases.

Different parameters, such as processing techniques, can modify the mechanical properties of silk fibers. In addition, silk has no immunogenicity or cytotoxic effects [56]. When some immunogenic reaction to silk is observed, it is attributed to the sericin proteins.

Processing Methods

Silk fibroin can be processed using different techniques for different architectures, as shown by Correia et al. [57]. They produced scaffolds using different solvents (aqueous versus hexafluoro-2-propanol [HFIP]), different pore sizes (250−500 versus 500−1000 μm), and different structures (lamellar versus spherical pores). To obtain aqueous-derived scaffolds with spherical pores, they used a salt leaching technique employing sodium chloride particles that were removed with water. In the case of aqueous-derived scaffolds with lamellar pores, they cast the silk solution in a tube, froze it, lyophilized it, and induced the formation of β-sheet by autoclaving the scaffolds. On the other hand, to produce HFIP-derived scaffolds, they used salt leaching technique as previously described and induced the β-sheet by freeze-drying it. The first technique resulted in pores sizes ranging from 140 to 250 μm, high interconnectivity (97%), and high porosity (87%) whereas the second resulted in smaller pore sizes (126 μm), lower interconnectivity (63%), and lower porosity (64%). Moreover, the HFIP-based silk scaffold had a higher stiffness than did the aqueous-derived scaffolds.

Sommer et al. compared two different processes, salt leaching and inverse opal, to obtain reproducible highly porous scaffolds (Fig. 32.5) [58]. Salt leaching resulted in scaffolds with pore sizes varying from 6 to 678 μm and 92% porosity, whereas inverse opal resulted in scaffolds with pores sizes varying from 6 to 312 μm and 84% porosity.

Wang et al. used electrospinning to produce fibers about 167 nm in diameter but with a low tensile strength [59]. They also blended silk with poly(lactide-*co*-ε-caprolactone) to obtain higher fiber diameters (≈250 nm) and higher tensile strength, which showed improved cellular interactions in vitro and enhanced new bone formation in vivo.

3D plotting allows the development of scaffolds with a controlled hierarchical structure with different pore sizes, which facilitates cell attachment, proliferation, vascularization, and the delivery of nutrients and physical cues. Xu et al. fabricated hierarchically porous scaffolds using 3D plotting and freeze-drying [60]. The obtained scaffold had a first level with 1-mm pores and a second level with approximately 50- to 100-μm pores. This hierarchical structure increased cell attachment four times and enhanced cell proliferation, ALP activity, and bone-related gene expression compared with no hierarchical structures.

Silk Fibroin in Bone Tissue Engineering Applications

Silk fibroin is a biomaterial with attractive features for bone tissue engineering. Correia et al. showed that higher stiffness resulted in higher osteogenic induction and the formation of bone-like tissue [57]. Although the mechanical properties were inferior in developed silk scaffolds compared with those in decellularized trabecular bone scaffolds, the cellular activities were similar. Finally, they suggested that lamellar pores were beneficial for differentiation into lamellar bone, whereas spherical pores led to the development of woven bone.

Nevertheless, it is challenging to obtain scaffolds with mechanical properties that meet the high compressive properties of bone. Mandal et al. developed a high−compressive strength polymeric bone composite based on

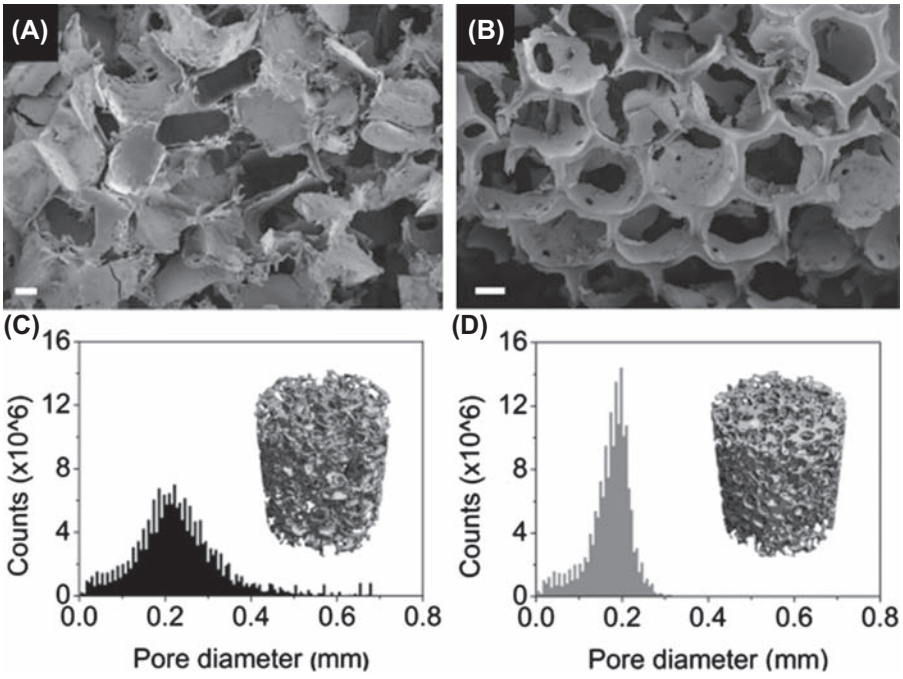

FIGURE 32.5 Scanning electron micrographs of silk scaffold pores produced by: (A) salt-leached process, or (B) inverse opal (scale bar = 100 μm). Pore diameter distribution of: (C) salt-leached scaffolds and (D) inverse opal scaffolds. *Adapted with permission Sommer MR, Vetsch JR, Leemann J, Muller R, Studart AR, Hofmann S. Silk fibroin scaffolds with inverse opal structure for bone tissue engineering. J Biomed Mater Res B Appl Biomater 2016.*

silk protein−protein interfacial bonding [61]. For that, they produced a micron-sized silk fiber and used it as filler for a silk matrix, increasing the mechanical properties 50- to 100-fold compared with control HFIP−silk scaffolds. The result, which was associated with surface roughness and porosity, favored stem cell differentiation into osteoblasts in vitro. In addition, a minimal in vivo immunomodulatory response was observed.

Using a different strategy, Yan et al. developed silk/nanosized calcium phosphate to improve silk scaffolds for bone tissue engineering [62]. The promising results had improved mechanical properties, architecture, and stability, bioactivity, and no cytotoxicity. Later, the authors conducted in an in vivo study in which the developed scaffolds promoted new bone formation [63]. The group demonstrated the use of a bilayer construct composed of a silk scaffold and a silk/nanosized calcium phosphate scaffold is a promising candidate for osteochondral defect regeneration [64].

To mimic the composition of bone, silk was mixed with HAp, the primary mineral found in bone, to produce an osteoinductive composite. HAp not only increased the compressive strength of the scaffolds, it enhanced the growth and osteogenic capability of cells [65].

Nanofibrous silk scaffolds also showed promising results owing to their similarity with the native ECM. The silk-based nanofibrous scaffolds facilitated cell proliferation and osteogenic differentiation in vitro [59]. Furthermore, in vivo, they promoted new bone formation, demonstrating their potential for bone tissue regeneration.

STARCH

Starch is naturally produced by plants and algae in the form of granules as an energy-storing compound. These granules have different sizes (1−110 μm) and composition depending on the source. There are two types of starch: transitory and storage. The first is produced during photosynthesis in leaves and is rapidly degraded; the second is stored for longer periods in seeds or roots [66].

This biodegradable and inexpensive natural polymer is composed of two types of α-glucan polymers, linear poly(1,4-β-D-glucopyranose) (amylose) and branched poly(1,4-β-D-glucopyranose) with branches of (1,6-D-glucopyranose) (amylopectin) occurring in nearly every 25 glucosidic moieties [66].

Starch is the main carbohydrate source in the human diet. It is degraded by the action of amylases, which act on α-1,4-glycosidic bonds. The degradation products are oligosaccharides that can be metabolized to produce energy.

Native starch can be recognized as waxy starch with a low amylose content, normal starch with 15%–30% amylose, and high amylose with more than 50% amylose. The crystallinity of these can vary from 50% in waxy starch to 15% in high-amylose starch [67].

To process starch using different methods and allow its use in various applications, it is necessary to disrupt and melt its semicrystalline granular structure, i.e., gelatinization. During gelatinization there is the diffusion of water into the interior of the granules, producing swelling and consequently disruption of the crystalline structure. The amount of amylose is inversely related to the energy necessary to initiate gelatinization. In this sense, high-amylose starch needs less heat to initiate gelation compared with waxy starch.

Starch is usually brittle, which limits its use in most applications. Thus, to improve the properties of starch, it needs to be modified or blended with other polymers.

Processing Methods

Because of the thermoplastic behavior of starch-based blends and composites, it is possible to produce scaffolds using traditional solvent casting methodologies [68]. For that, starch-based blends are dissolved in chloroform, cast onto a mold, and allowed to dry. In a different approach, blends of starch and cellulose were processed by combining solvent casting, salt leaching, and freeze-drying [69]. The obtained scaffolds had a porosity between 20% and 50%, depending on the components. The porosity increased with an increase in the amount of components. The authors also showed that adding cellulose promoted an increase in tensile properties, as demonstrated by the increase in Young's modulus and tensile strength. However, a decline in the compression modulus and compression strength was observed.

A different study used a supercritical assisted phase-in version process because it allowed control over the scaffolds' structure by fine-tuning the temperature and pressure [70]. In this case, the polymer solution was placed into a mold and immersed in a nonsolvent bath, which originated the phase separation under controlled temperature and pressure. The scaffolds had 64% porosity and 65% interconnectivity at 45°C and 80 bar. However, at 55°C and 150 bar, the scaffolds had a more compact structure with low interconnectivity (Fig. 32.6).

Wet spinning was also used to produce fibers of starch mixed with other polymers, such as poly(ε-caprolactone) [68,71]. This technique consisted of extruding a polymer solution loaded into a syringe into a coagulation bath (e.g., a

(A)

Experiment	*P* (bar)	T (°C)	Porosity (%)	Interconnectivity (%)
1	80	45	64	65
2	100	45	75	67
3	150	45	53	40
4	150	55	62	23

(B)

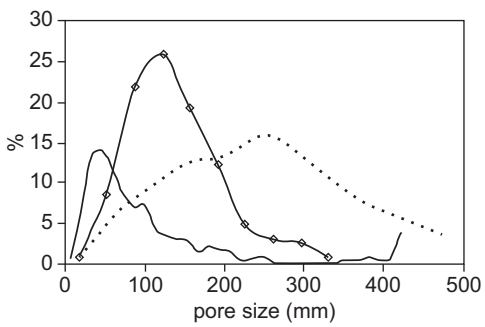

FIGURE 32.6 Effect of pressure (*P*) and temperature (T) on (A) porosity and interconnectivity in starch–poly(ε-caprolactone) scaffolds, and (B) effect of pressure on the pore size distribution at 45°C (*diamond* = 80 bar; *dashed line* = 100 bar; *solid line* = 150 bar). *Adapted with permission Duarte AR, Mano JF, Reis RL. Supercritical phase inversion of starch-poly(epsilon-caprolactone) for tissue engineering applications. J Mater Sci Mater Med. 2010;21(2):533–40. Copyright © 2009, Springer Science + Business Media, LLC.*

methanol or calcium silicate solution). The fibers' thickness was approximately 100–200 μm and the final scaffolds had a porosity of 60%–85%, depending on the coagulation solution used. When methanol was used, the porosity was higher than in the case of the calcium silicate solution [71].

A different approach was pursued to obtain fibers without using solvents, which can be risky for tissue engineering [72]. In that study, fibers were prepared using melt spinning followed by fiber bonding. The first step consisted of extruding the polymer at 150°C into a bath at 13°C. The fibers produced using this method were 120–500 μm in diameter. Then, to prepare the scaffolds, the fibers were chopped and placed into a mold and heated to 120°C. In a further step, pressure was gently applied to avoid crushing the fibers and to maintain porosity. The scaffolds produced using this method had about 75% porosity and 97% pore interconnectivity, in which the pore size was around 275 μm [72].

Furthermore, water-in-oil emulsification can be used to obtain starch–chitosan microparticles [73]. The microparticles' scaffolds had a pore size of 80–150 μm.

In a different study, nanocomposites of starch, HAp, and chitosan were produced using coprecipitation [74]. For that, a solution of starch and chitosan was prepared, followed by the addition of calcium nitrate and 3-deoxy-D-arabinoheptulosonate 7-phosphate synthase to promote the nucleation of HAp. Finally, the produced composites were allowed to dry at 85°C. The porous scaffolds obtained had a compressive strength of about 9 MPa, which is in the range of the compressive strength of trabecular bone.

Starch in Bone Tissue Engineering Applications

A number of studies used starch-based scaffolds for bone tissue engineering. Scaffolds composed of starch with polycaprolactone and stem cells improved bone regeneration upon implantation into a critical-sized defect [75]. In one study, several stages of osteoblastic differentiation were tested to improve bone regeneration when seeded on starch and polycaprolactone scaffolds [76]. The authors observed that scaffolds loaded with osteoblastic cells had a higher amount of new bone formation compared with scaffolds loaded with no differentiated cells. In addition, blood vessels were observed in the periphery of the scaffold with and without cells, which indicated that this strategy promoted not only new bone tissue formation but also angiogenesis.

In an improved approach, Leonor et al. modified scaffolds of starch and polycaprolactone with silanol groups [71]. Silanol groups were reported to promote osteoblast differentiation, which is an interesting feature for bone tissue engineering strategies. The developed scaffolds showed that the silanol functional groups promoted the differentiation of stem cells into osteoblasts.

Requicha et al. produced a double-layer scaffold for use in periodontal defects, composed of one membrane layer and a 3D scaffold layer [68]. The membrane layer prevents the ingrowth of the gingival epithelium into the periodontal defect and promotes the recruitment and adhesion of native cells. The 3D scaffold layer supports new bone tissue ingrowth. The assay showed that stem cells were able to adhere and proliferate, and that in the presence of silanol groups, they were able to express osteogenic markers. Taking into consideration these promising results, the authors tested the developed double scaffold in vivo in a mandibular rodent model [77]. After 8 weeks of implantation, the scaffolds had higher new bone formation than did the collagen control scaffolds. In addition, no proliferation of bone tissue was observed along the membrane layer, which indicated the success of the design of the first layer for preventing the ingrowth of tissue. However, no differences were observed between scaffolds with or without silanol groups.

NATURAL-BASED BIOCERAMICS

Bioceramics are used in tissue engineering as solid scaffolds (e.g., reconstruction of ear ossicles), powders (e.g., bone filling), and porous scaffolds (e.g., bone regeneration). Among those, bioceramics can be divided into nearly inert (e.g., zirconia), bioactive (e.g., bioactive glass), and resorbable (e.g., calcium phosphates). The first type, nearly inert, is not usually chosen for regenerative medicine because it promotes the formation of a fibrous capsule instead of tissue regeneration. The other two types, bioactive ceramics and resorbable ceramics, have been considered promising materials for bone regeneration and have undergone clinical trials. For example, calcium phosphate has been studied as a synthetic bone graft for donor site repair [78], and silica-based bioactive glasses have been used for the rehabilitation of canal-wall-down mastoidectomies [79]. Here, we will highlight the routes and natural origin sources for obtaining these materials and review their application in bone tissue engineering strategies.

CALCIUM PHOSPHATES

Calcium phosphates are crystalline ceramics with a structure and chemical composition similar to minerals found in bone. An example of calcium phosphate widely used is HAp, which is represented by the chemical formula ($Ca_{10}[PO_4]_6[OH]_2$); it has a structure similar to the main mineral found in bone, apatite.

HAp is available as a bone filler and as coatings on prostheses, owing to its good biocompatibility, bioactivity, high osteoconductive and/or osteoinductive capacity, nontoxicity, noninflammatory behavior, and nonimmunogenic properties [80]. Calcium phosphate scaffolds are described as promoting osteogenesis and osteointegration, which seems to be related to surface charge and the chemistry and topography of the scaffolds. However, this ceramic has a low resorption rate. Therefore, other calcium phosphates, such as β-tricalcium phosphate, which have fast resorption rates, have been studied. As an alternative, new sources of calcium phosphate have been explored (Fig. 32.7). Calcium phosphates can be extracted from different marine origins such as fish bones, corals, seashells, and algae. Sponges from the phylum Porifera have been explored because their skeleton is composed of bioceramics [81], and new methods have been developed to assess the overall regenerative and mineralogenic performance in zebrafish [82]. HAp has been extracted from the porous exoskeleton of corals owing to its similarity to bone and its mechanical, osteoconductive, and resorbable properties. This interesting material can be found commercially, such as in Pro Osteon 200R (Biomet), an osteoconductive bone graft.

Another marine source that is widely used is shells, which are mainly composed of calcium carbonate associated with approximately 2% polysaccharides and glycoproteins. Eggshells are also an interesting source for obtaining HAp [83]. Eggshells are an easily obtained, low-cost resource because they come from food waste and the HAp can be readily extracted by calcination and milling.

Synthetic HAp can be produced by different methods [1]: hydrothermal (in the presence of water and at a high temperature and pressure) [2]; sol–gel (formed by apatite crystals at a low temperature and pressure owing to the high reactivity of calcium and phosphorus reagents used) [3]; wet chemical (by mixing an aqueous precursor solution or hydrolyzing calcium phosphate); and [4] biomimetic deposition (the nucleation of HAp when subjected to

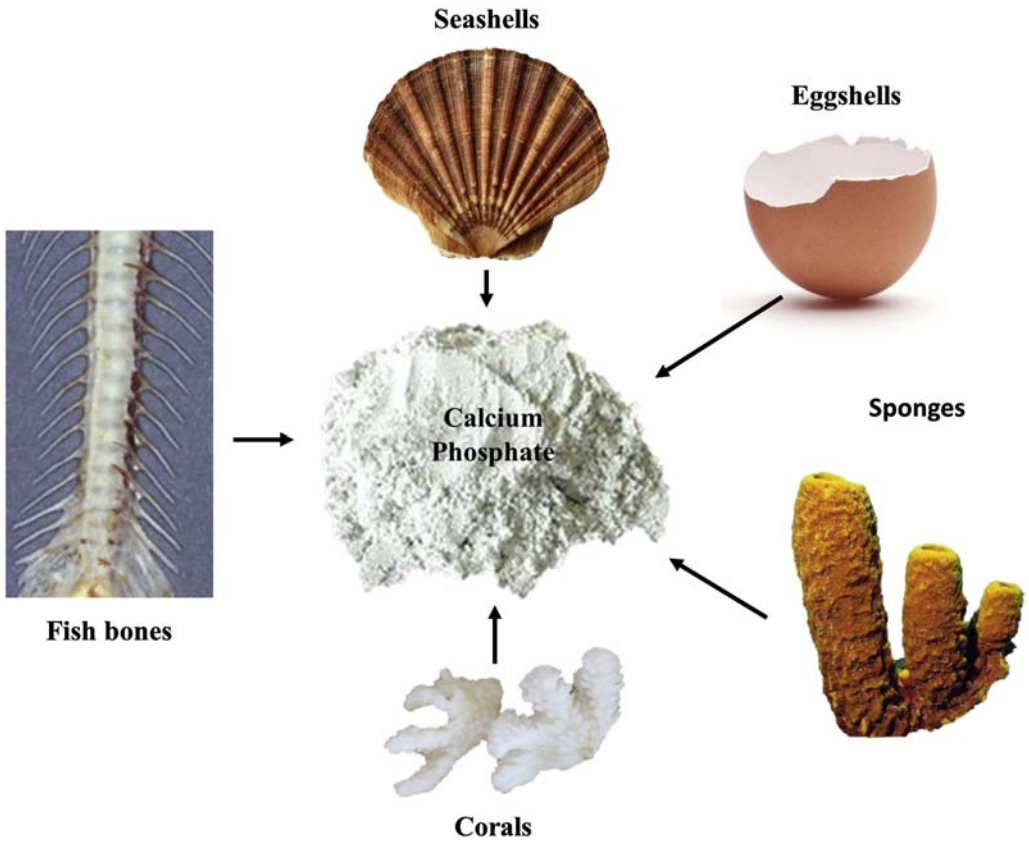

FIGURE 32.7 Some sources of calcium phosphate.

body fluids) [84]. In addition, parameters such as the pH, reaction time, temperature, and concentration of reagents during synthesis can influence the obtained HAp.

Apart from the differences observed caused by the different extraction or production method and origin, natural HAp had better outcomes than synthetic HAp [83]. This could be the result of significant differences observed between naturally derived HAp and synthetic HAp.

Processing Methods

HAp forms a bond with the host tissue. This feature has made this material the first choice for preparing scaffolds for bone tissue engineering.

HAp scaffolds can be obtained by foam replication, which requires the use of a polymeric mold and allows control over the scaffolds' structure. For example, polyurethane sponges can be used as templates [85]. During this procedure a suspension of HAp is mixed with porous synthetic polymeric foams until the pores are totally filled and sintered, allowing the development of a porous scaffold with a porosity of about 70%. The obtained pore size varies between 100 and 300 μm and the pore wall has a thickness of 50 μm. Moreover, it was shown that the pores are uniformly distributed. The compressive strength of the prepared scaffolds, 1.3 MPa, is close to that of cancellous bone.

Rapid prototyping technologies are being used to produce scaffolds with precise morphology (e.g., shape, porosity, and pore size). Control over mechanical properties, biological effects, and degradation kinetics is associated with control of the scaffold architecture. Furthermore, that approach can enable the development of dimensionally accurate prototypes of bone, which is an attractive feature for bone engineering. Solid free-form fabrication allows the production of inorganic complex-shaped scaffolds directly from 3D objects fabricated using computer-aided design [86]. For that, an HAp slurry is produced by mixing HAp powder with a freezing vehicle (e.g., camphene). Then the slurry is placed into a mold previously produced using a stereolithography device and allowed to cool. Finally, it is freeze-dried to remove the freezing vehicle and sintered to remove the mold and any freezing vehicle still present, which densifies the scaffold. This method allows the production of pores 100 μm in diameter. A similar approach that creates custom scaffolds is 3D printing [87]. The scaffold can be produced with complex internal structures and high resolution. Scaffolds prepared by this method had smaller pores (10−60 μm) and a porosity of about 50%. In addition, the compressive strength (0.88 MPa) was close to the lower limit of cancellous bone.

Gas foaming, in which the production of gas allows the development of pores, was used to produce an inorganic porous matrix [88]. Hydrogen peroxide was mixed with a ceramic slurry, which generated gas after heating. Then, the slurry was molded, dried, and sintered, resulting in a scaffold with interconnected pores and 75% porosity. The pore size ranged from 200 to 400 μm.

Franco et al. produced fibers of HAp of submicron dimensions using electrospinning [89]. They took advantage of the gel phase produced during the sol−gel technique for HAp production. At the end, the fibers were sintered. This technique produced fibers 122 nm in diameter for the lower viscosity tested and 567 nm for the higher viscosity.

Calcium Phosphate in Bone Tissue Engineering Applications

Scaffolds should meet some criteria to enable tissue regeneration. In addition to being biocompatible and porous (minimum pore size of about 100 μm for tissue ingrowth), scaffolds should be osteoconductive and osteoinductive, which are well-known features of HAp scaffolds. Moreover, some issues need to be overcome to fulfill all of the criteria, such as the ability to fine-tuning biodegrading and to improve mechanical strength. Tripathi et al. studied new methods for scaffold preparation that allowed scaffold architecture to be tailored [85]. Using sponges as a template, they successfully obtained scaffolds that enabled the attachment and differentiation of human osteoblasts.

Bone tissue is composed not only of HAp but also of collagen type I. To mimic bone composition, Changchun et al. developed a scaffold with a hierarchical structure composed of calcium phosphate, collagen, and HAp [88]. These scaffolds demonstrated better mechanical strength than scaffolds composed solely of calcium phosphate, and had good biocompatibility. In addition, the authors observed better osteoinductivity and increased bone formation in vivo compared with calcium phosphate scaffolds and calcium phosphate with collagen scaffolds. Thus, the developed scaffolds composed of calcium phosphate, collagen, and HAp are a promising material for bone engineering.

The mechanical properties of synthetic HAp scaffolds are still in the lower limit of mechanical properties presented by natural bone. Therefore, Nandi et al. pursued the possibility of HAp derived from natural sources such as marine corals, which are described as exhibiting mechanical properties and porosity similar to those of natural bone [90]. They developed coralline HAp scaffolds with growth factors to enhance the formation of bone tissue.

Upon mechanical evaluation, the authors observed adequate compressive strength values for coralline HAp scaffolds. Moreover, the in vivo assessment of scaffolds loaded with growth factors showed higher new bone formation compared with scaffolds without growth factors.

SILICATE CERAMICS

Silicate ceramics can be found in the skeleton of some marine sponges. This inorganic polymer is produced by an enzymatic reaction in siliceous sponges during the formation of the skeleton. Bioactive silicate ceramics have been studied for bone tissue engineering owing to their ability to induce the formation of an apatite layer on their surface. The apatite layer stimulates cell differentiation along the osteoblastic lineage and favors the adhesion of stem cells [91]. Silicate ceramics are described as enabling osteointegration with host bone. More than 20 different silicate ceramics have been developed with fine-tuned mechanical properties, bioactivity, and degradation rates. However, the low mechanical properties of this material have limited its application as a filler or coating for metal implants. Generally, silicate ceramics allow cell adhesion, but differences in chemical composition result in different adhesion rates. Their composition influences surface degradation, which could influence cell adhesion.

Among the silicate ceramics, the most well-studied is silicate-based bioactive glass 45S5, also known as Bioglass [92]. One of the most attractive characteristics of bioactive glass is the release of soluble ions during degradation, which that can induce bone formation [92]. Furthermore, bioactive glass 45S5 promotes the secretion of angiogenic growth factors in vitro and angiogenesis in vivo [91]. However, its use in clinics is limited and it is employed less often than calcium phosphates. Different limitations have been pointed out to explain this. For example, bioactive glass has slow degradation rates, which limit bone growth [91], and the preparation of porous scaffolds is challenging [92]. Some features of bioactive glass, such as brittleness and the promotion of nucleation, have prompted its use for improving the mechanical properties and bioactivity of other materials [40].

Processing Methods

Bioactive glass can be obtained through different techniques. The most widely used is the sol−gel process, which is composed of the formation of an inorganic network [93]. For that, metal alkoxides in solution are mixed and further hydrolyzed and gellified. Finally, they are submitted to low-temperature firing to produce a glass phase and then dried, sintered, and fractured. This method obtains pores with a diameter between 6 and 18 nm. A different method is the melt-derived approach, in which the glass components are mixed, melted, and homogenized at high temperature [96]. The melted solution is poured into a liquid and then cool and cracked into small pieces to obtain a powder.

Porous scaffolds of bioactive glass have been developed through different methods to adjust the pore size and interconnectivity, such as foaming [94]. This method is composed of mixing the ceramic slurry with the polymeric foam, which is eliminated at the end of the process. With this approach, the foam determines the final scaffolds' architecture, allowing pore sizes to be controlled.

Particulate leaching is a different method also used to produce glass scaffolds [95]. In this case, porogen particles are mixed with the ceramic slurry and leached out during the sintering step. The resulting scaffold has an interconnected network.

A different method is the melt-derived approach [96]. This approach consists of mixing the glass components followed by melting and homogenizing them at high temperature. Afterward, the melted components are placed into a mold to obtain the desired scaffold. A different approach is to use the melt glass to produce fibers, a technique known as melt spinning. For example, Deliormanli et al. prepared a solution of bioactive glass by mixing different precursors (tetraethyl orthosilicate, triethyl phosphate, calcium nitrate tetrahydrate, potassium nitrate, magnesium nitrate hexahydrate, and sodium nitrate) with aqueous PVA [97]. Then, this solution was electrospun under an applied voltage, aged at 60°C, allowed to dry at 120°C, and finally sintered.

Silicate in Bone Tissue Engineering Applications

As mentioned, silicate ceramic scaffolds fulfil many of the criteria of an ideal scaffold, but they are brittle. Therefore, new strategies have been pursued to improve they mechanical characteristics of these scaffolds, such as fabricating composite scaffolds composed of an inorganic and an organic phase. Moreover, some studies have used this

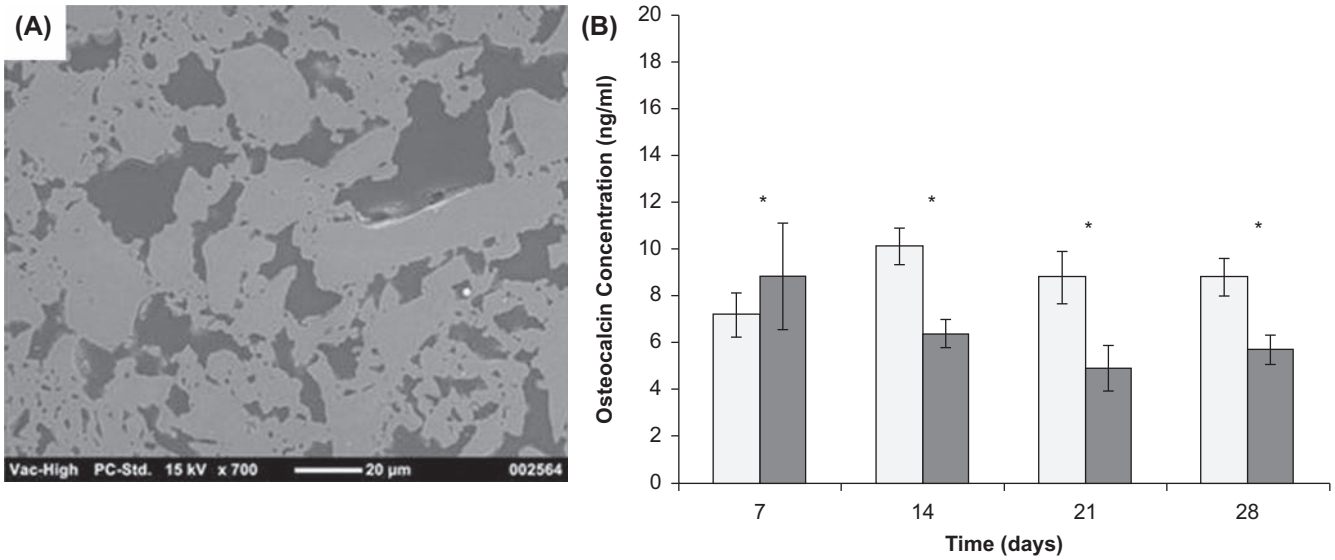

FIGURE 32.8 Effect of silicate-substituted calcium phosphate with enhanced porosity versus Bioglass on osteogenic marker expression. (A) Scanning electron microscopy image of silicate-substituted calcium phosphate with enhanced porosity. (B) Osteocalcin expression comparing silicate-substituted calcium phosphate with enhanced porosity (*light bars*) versus Bioglass (*dark bars*) (Magnification ×700). *Adapted with permission De Godoy RF, Hutchens S, Campion C, Blunn G. Silicate-substituted calcium phosphate with enhanced strut porosity stimulates osteogenic differentiation of human mesenchymal stem cells. J Mater Sci Mater Med 2015;26(1):5387. Copyright © 2015, Springer Science + Business Media New York.*

approach to improve the mechanical properties of other materials such as hydrogels [40]. Gantar et al. reinforced gellan gum hydrogels with bioactive glass nanoparticles and observed an improvement in mechanical properties and bioactivity. In addition, the developed composite sponge-like hydrogels allowed stem cell adhesion and spreading, showing great promise for bone tissue engineering. In a different study, bioactive glass was mixed with chitosan, promoting the enhancement of the scaffolds' mechanical properties [12]. The improvement in mechanical properties resulted from the formation of an apatite layer upon body fluid immersion.

In in vivo studies, bioactive glass was loaded with growth factors to promote repair of bone defects [98]. Upon implantation of the designed scaffolds into critical size defects, a bridging callus was observed only in scaffolds loaded with the growth factor. In a study conducted by Meretoja et al., poly(ε-caprolactone/D,L-lactide)-based scaffolds were filled with bioactive glass and implanted ectopically [99]. After 12 weeks, the authors showed that the developed scaffolds were able to induce ectopic bone formation.

Silicate-substituted calcium phosphate with enhanced porosity was developed by De Godoy et al. and compared with Bioglass in vitro [100]. Higher cell adhesion and enhanced cell differentiation along the osteogenic lineage were observed in cells cultured in silicate-based scaffolds, compared with Bioglass. The obtained results showed that the microstructure and chemistry of the scaffold influence cells behavior (Fig. 32.8).

CONCLUSIONS

The unique properties of natural-based materials have attracted the interest of many scientists, with the purpose of repairing and regenerating damaged bone tissue. Bioavailability, the possibility of blending, and the processability of each material guarantee its use, alone or in combination, in the continuous pursuit of an ideal approach to obtaining an efficient therapy. However, natural-based materials have some limitations that must be overcome. Although the future of bone tissue regeneration is promising, much work must be done, namely in what concerns to the need of promote the vascularization of implants when large bone defects regeneration is envisioned. The continuous development of new and personalized technologies such as high-resolution bioprinting has provided new tools that allow the exploration of the potential of natural-based materials in the clinic. At the same time, these new tools and other advanced processing technologies have encouraged the creation of personalized and safer medical devices, which may enhance their success rate and faster translation to the clinics. In addition, growing knowledge in the most diverse fields, such as an increasing understanding of interactions between biological tissues and materials, will boost progress of new strategies. Ultimately, this progress will result in the optimization of the performance of natural-based materials and their successful translation to clinics.

List of Acronyms and Abbreviations

3D three-dimensional
ALP alkaline phosphatase
ASCs adipose derived stem cells
ECM extracellular matrix
GAGs glycosaminoglycans
HAp hydroxyapatite
HFIP hexafluoro-2-propanol
MSCs mesenchymal stem cells
PHAs polyhydroxyalkanoates
PHB poly 3-hydroxybutyrate
PHBHHx poly 3-hydroxybutyrate-co-3-hydroxyhexanoate
PHB-HV polyhydroxybutyrate-hydroxyvalerate
PHBV poly 3-hydroxyvalerate
PHV polyhydroxyvalerate
PVA polyvinyl alcohol
rhBMP-2 human bone morphogenetic protein-2
β-TCP β-tricalcium phosphate

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CHAPTER

33

Synthetic Polymers

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INTRODUCTION

Regenerative medicine is an emerging interdisciplinary approach to repairing or replacing damaged or diseased tissues and organs. To reestablish tissue and organ function impaired by disease, trauma, or congenital abnormalities, regenerative medicine employs cellular therapies, tissue engineering strategies, and artificial or biohybrid organ devices. Typically, these techniques rely on combinations of cells, genes, morphogens, or other biological building blocks with bioengineered materials and technologies to address tissue or organ insufficiency.

Materials used in these approaches range from metals and ceramics to natural and synthetic polymers as well as their microcomposites and nanocomposites. When used in a three-dimensional context, these materials are processed into microporous and/or nanoporous cell carriers of various structures and properties, typically called scaffolds, a topic discussed elsewhere in this book. This chapter focuses exclusively on synthetic polymers used in regenerative medicine. Some synthetic derivatives of natural materials are briefly discussed where appropriate. In addition to the various facets of regenerative medicine, a plethora of synthetic polymers with different compositions and physicochemical properties have been developed and investigated; however, research is ongoing. Synthetic materials have an important key role in many applications of regenerative medicine, including implants, tissue engineering scaffolds, and orthopedic fixation devices. In a broader sense, sutures, drug delivery systems, nonviral gene delivery vectors, and sensors made from synthetic polymers are further examples.

This chapter provides a structural overview of these synthetic polymers and discusses their physicochemical characteristics, structure–property relationships, applications, and limitations. Synthetic polymers that are hydrolytically labile and erode (biodegradable polymers) are considered, as are as those that are bioinert and remain unchanged after implantation (nondegradable polymers). It is the authors' intention to provide a thorough overview of available classes of synthetic material. Some polymer classes are briefly mentioned and their chemical structures are provided; other more relevant materials are discussed in greater detail. For most polymer classes and properties, reviews are referenced for guidance to further reading.

In general, the history of biomaterials can best be organized into four eras: prehistory, the era of the surgeon hero (first-generation biomaterials), designed biomaterials and engineered devices (second-generation biomaterials), and the contemporary era leading into the new millennium (third-generation biomaterials) [1,2]. As far back as AD 600, the use of dental implants made from materials such as seashells or iron was reported. Also, there is evidence that sutures have been used to close large wounds for as long as 32,000 years. The word "biomaterials," however, was introduced within the half century. Almost at the same time, aided by rapid advancements in industrial polymer development and synthesis, the exploration of synthetic polymers for biomedical applications began. The development of plastic contact lenses, using primarily poly(methyl methacrylate) (PMMA), started around 1936, and the first data on the implantation of nylon as a suture were reported in 1941. This development was accompanied by studies on the biocompatibility of the new materials. From the beginning, differences in foreign body reactions to the materials became apparent. Additives such as plasticizers, unreacted monomers, and degradation products were discussed as possible causes leading to an awareness of polymer's quality for biomedical applications and biocompatibility testing.

At the end of World War II, a wide variety of durable, high-performance metal, ceramic, and especially polymeric materials was available, inspiring surgeons to break new ground in replacing diseased or damaged body parts.

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Materials including silicones, polyurethanes (PUs), Teflon, nylon, methacrylates, titanium, and stainless steel were available "off the shelf" for surgeons to apply to medical problems [2]. Primarily medical and dental practitioners, driven by the vision to replace lost organ or tissue functionality, used minimal regulatory constraints to develop and improvise replacements, bridges, conduits, and even organ systems based on such materials. Those pioneering approaches laid the foundation for novel procedures and engineered biomaterials. Such early implants made from industrial materials available "off the shelf" were often poorly biocompatible, in many cases owing to insufficient purity. With a developing understanding of the immune system and foreign body reactions, the first generation of materials was developed during the 1960s and 1970s by engineers and scientists for use inside the human body. The primary goal of early biomaterial development was to achieve a suitable combination of physical properties to match those of the replaced tissue with a minimal toxic response in the host [3]. After this paradigm, more than 50 implanted devices made from 40 different materials were in clinical use in 1980. In the early 1980s, research began to shift from materials that exclusively exhibited a bioinert tissue response to materials that interacted actively with their environment. Another advance in this second generation was the development of biodegradable materials that exhibited controllable chemical breakdown into nontoxic degradation products that were either metabolized or directly eliminated. Biodegradable synthetic polymers were designed to resolve the interface problem, because the foreign material is ultimately replaced by regenerating tissues and eventually the regeneration site is histologically indistinguishable from the host tissue. By 1984, resorbable polymers were routinely used clinically as sutures. Other applications in fracture fixation aids or drug delivery devices quickly emerged. Despite the considerable clinical success of bioinert, bioactive, and resorbable implants, there is still a high long-term prosthesis failure rate and the need for revision surgery [2].

Improvements in first- and second-generation biomaterials have been limited for one main reason: unlike living tissue, artificial biomaterials cannot respond to changing physiological loads or biochemical stimuli. This limits the lifetime of artificial body parts. To overcome these limitations, a third generation of biomaterials is being developed that involves the molecular tailoring of resorbable polymers for specific cellular responses. By immobilizing specific biomolecules such as signaling molecules or cell-specific adhesion peptides or proteins onto a material, it is possible to mimic the extracellular matrix (ECM) environment and provide a cell-adhesive surface [1,4–6]. Biomimetic surfaces are promising tools for controlling cell adhesion, integrating implants, differentiating cells, and developing tissues. Synthetic polymer matrices can also be tailored to deliver drugs, signaling molecules, and genetic code and thus provide versatile technologies for regenerative medicine [7–9]. Constantly expanding knowledge of the basic biology of stem cell differentiation and the corresponding signaling pathways as well as tissue development provides the basis for the molecular design of scaffolds. In attempts at tissue engineering, which aim to regenerate lost or defective tissue by transplanting in vitro engineered tissue constructs based on a patient's own cells, one no longer strives to match scaffold mechanical properties closely to those of the replaced tissue. Instead, it is considered important for the engineered transplanted construct to be steadily remodeled in vivo to resemble the histological and mechanical properties of the surrounding tissue [10]. Owing to this paradigm shift, mechanically labile hydrogels, especially injectable systems that can be used to encapsulate cells directly, have gained great importance as the basis for biomimetic cell carriers. Hydrogels are characterized by a high content of water that allows encapsulated cells to survive and enables the sufficient passive transport of nutrients, oxygen, and waste. Hydrogel-forming materials typically offer functional groups for chemical modifications, and their degradation can be controlled by chemical composition and cross-linking content.

After a brief overview on synthesis techniques, inert and biodegradable synthetic polymers representative of all three generations will be presented in subsequent sections. Their structure, synthesis, physicochemical properties, and applications will be described.

POLYMER SYNTHESIS

Polymerization reactions for the synthesis of organic polymers are often categorized into chain-growth polymerizations and step-growth polymerizations, depending on how the chemical process of chain formation proceeds. The synthesis of polymers with a carbon–carbon backbone such as polyolefins and polyacrylates typically follows a chain-growth mechanism [11]. Chain-growth polymerizations involve the steps of chain initiation, chain propagation, and termination. Characteristics of this type of polymerization are that chain growth occurs only by the addition of monomers to the active chain end, generally at a high speed, and that only the monomer and polymer are present during the reaction. Depending on the nature of the reactive center of the propagation chains, chain-growth reactions are subdivided into radical, ionic (anionic or cationic), or transition-metal mediated (coordinative

or insertion) polymerizations. Suitable monomers contain an unsaturated carbon–carbon bond (double or triple) or are cyclic molecules with a sufficiently high ring strain. For the industrial synthesis of polyolefins, for example, free radical and transition-metal mediated polymerizations are commonly employed. Unlike radical polymerization, transition-metal coordinated mechanisms, such as with Ziegler–Natta catalysts, allow for the control of polymer tacticity [12]. A milestone in chain-growth polymerization history was the development of controlled or living radical polymerization techniques that allow for the precise control of polymer composition and architecture and yield polymeric products with low polydispersity [13].

Polymers that contain heteroatoms in the main chain are typically synthesized by a step-growth mechanism. During step-growth, the polymer molecular weight increases through the reaction of any two molecular species, i.e., monomers, oligomers, and polymer chains. In contrast to chain-growth, monomers disappear early on during the reaction and the polymer molecular weight slowly increases over the course of the reaction, which can last for days. Typical polymerization types that follow a step-growth mechanism are polycondensation and polyaddition reactions. In condensation reactions, small molecules such as water, alcohols, or hydrochloric acid are eliminated during step-growth. Polyethylene terephthalate and polyamides such as nylon and poly(propylene fumarate) [14] are examples of polymers that are synthesized by condensation reactions between carboxylic acid derivatives and diols or diamines (nylon). Most polyanhydrides are also synthesized by polycondensation reactions [15]. Polyaddition reactions follow a similar mechanism because nucleophilic groups react with electrophilic moieties during polymer chain buildup. In contrast to condensation reactions, addition reactions combine monomers without eliminating a small molecule. During PU synthesis, for example, diisocyanate monomers are reacted with diamines or dihydroxy-terminated molecules in the presence of catalysts under the formation of urethane and urea groups, respectively, to build up polymer chains [16].

Ring-opening polymerizations (ROPs) also yield polymers with heteroatoms in the main chain and are used to synthesize polyamines, polyethers such as poly(ethylene glycol)s (PEGs), and most biodegradable polyesters including polylactides, polyglycolides, and copolymers [17]. ROPs can follow chain-growth and step-growth kinetics and are executed in melts or solutions in the presence of catalysts and heat.

Driven by advances in drug design through combinatorial approaches in small-molecule chemistry, similar techniques have been adapted to polymerization chemistry [18]. Through the systematic screening of libraries of polymeric materials that have similar chemistries but are synthesized from a series of different monomers and comonomers in various combinations, structure–property relations can be identified and polymer properties can be fine-tuned for specific applications. Polymer properties that are screened using such approaches include the material's glass transition temperature, degradative properties, air–water contact angle, mechanical properties, cytocompatibility and cell proliferation.

NONDEGRADABLE SYNTHETIC POLYMERS

A common characteristic of most nondegradable synthetic polymers is their biological inertness [1]. These materials were developed to reduce the host response to the biomaterial to a minimum. Nondegradable synthetic polymers provide the basis for a plethora of medical devices as diverse as suture materials, orthopedic implants, fracture fixation devices, and catheters and dialysis tubing. These materials are also applied as implantable carriers for the long-term delivery of drugs, e.g., contraceptive hormones. Despite their excellent biological inertness and well-adjustable mechanical properties, orthopedic implants made from nondegradable synthetic polymers and nondegradable bone cements ultimately fail at a high rate from problems at the interface arising from a lack of integration with the surrounding tissue, infections, or bone resorption caused by stress shielding [19,20].

Major groups of nondegradable synthetic polymers are highlighted in the following section.

Polymers With a –C–C– Backbone

Polyethylene and Derivatives

Poly(ethylene), Poly(propylene), and Poly(styrene)

Poly(ethylene) (PE) (Fig. 33.1A), poly(propylene) (PP) (Fig. 33.1B), and poly(styrene) (PS) (Fig. 33.1C) are ubiquitous industrial polymers that have been applied as biomaterials. All three thermoplastic polymers are pure hydrocarbons and are synthesized by the direct polymerization of their corresponding monomers. Whereas PE can be synthesized by the radical or ionic polymerization of ethylene, special organometallic catalysts are required to

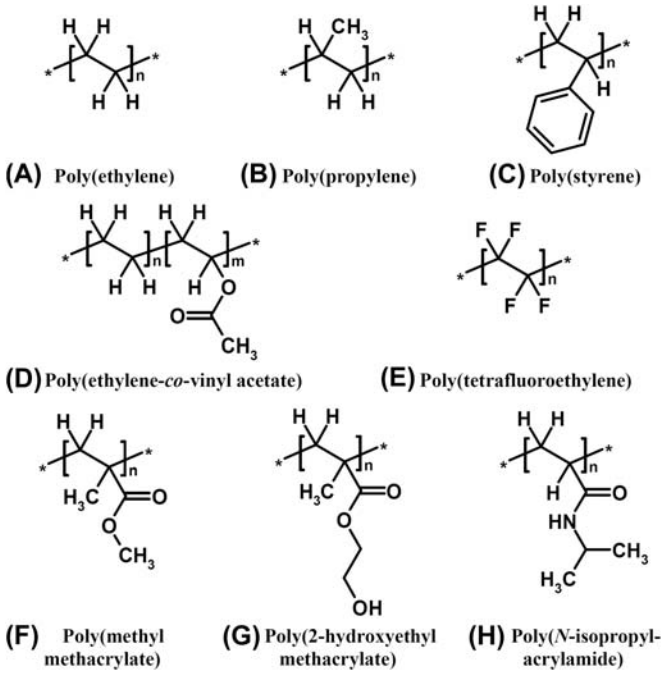

FIGURE 33.1 Chemical structures of nondegradable synthetic polymers (I).

polymerize propylene into useful PP. PE and PP are classified into several different categories based on their density, branching, and molecular weight. These parameters significantly influence the crystallinity and mechanical properties of the polymers. PE has been used to produce catheters. High-density PE, which is characterized by a low degree of branching and thus strong intermolecular forces and tensile strength, has been processed into highly durable hip prostheses. A three-dimensional fabric composed of PE fibers and coated with hydroxyapatite was used to regenerate hyaline cartilage in osteochondral defects in rabbit knees and showed successful biocompatibility [21]. The best-known application for PP is its use in syringe bodies. Copolymers of PE and vinyl acetate (poly[ethylene-*co*-vinyl acetate] [PEVAc]) (Fig. 33.1D) are widely used in nondegradable drug delivery devices [22]. PEVAc is one of the most biocompatible implant materials [23]; it has been approved by the US Food and Drug Administration (FDA) for use in implanted and topically applied devices.

Vitrasert , Implanon, and NuvaRing are examples of PEVAc-based drug delivery platforms [24–26] for ocular applications and long-term contraception. For the latter application, PE-based implants have also been developed.

PS is a hard, brittle polymer used to fabricate tissue culture flasks and dishes. By copolymerization with butadiene, copolymers with improved elasticity are synthesized that are used to make catheters and medical devices for perfusion and dialysis.

Poly(tetrafluoroethylene)

Poly(tetrafluoroethylene) (PTFE) (Fig. 33.1E), well-known as Teflon (DuPont), can be synthesized from liquid tetrafluoroethylene by radical polymerization and through the fluorination of PE. Among the known polymers, PTFE has the lowest coefficient of friction and excellent resistance to chemicals, and it is hemocompatible. Porous PTFE fiber meshes (Gore-Tex) have become a popular synthetic vascular graft material [27].

Poly(meth)acrylates and Polyacrylamides

Poly(meth)acrylate hydrogels are used in medical devices, especially for ocular applications (e.g., contact lenses and intraocular lenses), as drug delivery systems, and as cell delivery systems [28–30]. Three major types, PMMA, poly(2-hydroxyethyl methacrylate) (PHEMA), and poly(*N*-isopropylacrylamide), are discussed in more detail subsequently.

A variety of (meth)acrylate and acrylamide monomers with different functional groups are available; thus, poly(-meth)acrylates and polyacrylamides of different chemical compositions can be synthesized. Together with the free carboxylic acid moieties of (meth)acrylic acid, the presentation of different functional groups and charges along copolymer chains or within cross-linked hydrogels is possible. Using an imprinting technique, these moieties can

be oriented so that pouches are created that interact noncovalently with molecules, e.g., drugs or therapeutic peptides and proteins, via ionic interactions, hydrogen bonds, $\pi-\pi$ interactions, and hydrophobic interactions [31,32]. Besides intelligent hydrogels for controlled drug release, this technology has an impact on microfluidic devices, biomimetic sensors, intelligent polymeric membranes [33], and analyte-sensitive materials [34].

Poly(methyl Methacrylate)

PMMA (Fig. 33.1F) is a nondegradable polyacrylate and is the most commonly applied nonmetallic implant material in orthopedics. It was used as an essential ingredient to make dentures and then in the mid-1950s, PMMA was introduced for use in orthopedic surgery [35]. PMMA tissue biocompatibility became further apparent when Plexiglas fragments were accidentally implanted in the eyes and other body tissues of World War II fighter pilots during aircraft crashes.

PMMA can be polymerized in situ and cross-linked from a slurry containing PMMA and methyl (meth)acrylate monomers; thus, it is used as a common bone grafting material, mainly in the fixation of orthopedic prosthetic materials for hips, knees, and shoulders [36]. PMMA-based bone cements can be mixed with inorganic ceramics or bioactive glass to modulate curing kinetics and enforce mechanical properties. Antibiotics can be loaded within the cement to reduce the risk for prosthesis-related infection. Significant drawbacks of self-curing PMMA cements are that they are not degraded, that their high curing temperatures and toxic monomers can cause necrosis of the surrounding tissue, and that the cements have limited interactions with surrounding bone [37,38]. Therefore, the development of alternative injectable bone cements is directed toward biodegradable materials with improved curing properties and osteoconductive interfaces.

Because of its excellent biocompatibility and hemocompatibility and ease of manipulation, PMMA is used in many medical devices, including blood pumps and dialyzers. Its optical properties make it a candidate material for implantable ocular lenses and hard contact lenses [29]. PMMA also offers physical and coloring properties that are beneficial for denture fabrication [37].

Poly(2-hydroxyethyl Methacrylate)

PHEMA (Fig. 33.1G) was the first hydrogel to be successfully employed for biological use [39]. PHEMA has become the major component of most soft contact lenses and is also part of intraocular lenses [29]. Owing to their free hydroxyl groups, PHEMA gels contain relatively high amounts of water, which facilitates the diffusion of solutes and oxygen. PHEMA has excellent biocompatibility, which initiates the development of a plethora of hydroxyethyl methacrylate–containing copolymers. Hydrogels fabricated from PHEMA and copolymers have been intensively characterized for controlled drug delivery applications [40,41] and employed for biomedical uses. PHEMA gels, which have limited mechanical properties, have been used in attempts to reconstruct female breasts and nasal cartilages, and as artificial corneas as well as wound dressings [42].

In a subcutaneous rabbit model, porous PHEMA sponges promoted significant cellular ingrowth and neovascularization combined with good cytocompatibility [43]. A mineralization technique was demonstrated that exposed carboxylate groups on cross-linked PHEMA hydrogel scaffolds, promoting calcification [44].

Poly(*N*-isopropylacrylamide)

Poly(*N*-isopropylacrylamide) (PNiPAAm) (Fig. 33.1H) is important for injectable applications in drug and cell delivery using minimally invasive techniques because of its unique physicochemical properties [45]. PNiPAAm undergoes (lower critical) phase separation, resulting in the formation of an opaque hydrogel in response to a temperature above 32°C, the material's lower critical solution temperature (LCST). This thermoresponsive behavior is the result of strong hydrogen bonds between the polymer and water molecules and the specific molecular orientations of these bonds caused by the molecular structure of the polymer. The formation of hydrogen bonds between the polymer and the solvent lowers the free energy of the solution. Because of the hydrophobic *N*-isopropyl residues in PNiPAAm, the hydrogen bonds between water and the amide functionality require specific molecular orientation. Such ordered structures lead to negative entropy changes and positive contributions to the free energy. Because the enthalpic contribution to the free energy is temperature-dependent, the formation of strong but specifically oriented hydrogen bonds is no longer thermodynamically favored above a certain temperature. Consequently, PNiPAAm dissolves in water below the LCST. At and above the LCST, the polymer chains partially desolvate and undergo a coil-to-globule transition resulting in colloidal aggregation that may lead to gel formation or polymer precipitation [46,47]. Hydrogels formed by linear PNiPAAm at 32°C are instable and collapse substantially as the temperature is increased above the LCST. The synthesis of cross-linked networks and copolymers, typically with hydrophilic building blocks, has resulted in materials that demonstrate reversible thermogelation and form hydrogels with no

significant syneresis at body temperature. Different PNiPAAm-containing copolymers for cell delivery have been synthesized with acrylic acid, PEG, hyaluronic acid, and gelatin [45,48–50]. Detailed information is available for the in vitro and in vivo use of gelatin–PNiPAAm conjugates for the regeneration of articular cartilage [51,52]. A series of multifunctional *N*-isopropylacrylamide (NiPAAm)-based copolymers has also been developed [53]. One example is the injectable, thermosensitive macromer poly(NiPAAm-*co*-polylactide–hydroxyethyl methacrylate (HEMAPLA)–*co*-acrylic acid-*co*-*N*-acryloxysuccinimide) [54] that is composed of HEMAPLA for increased biodegradability via elevated backbone solubility upon PLA hydrolysis and reactive succinimide esters for the cross-linking of, e.g., type I collagen. Further examples of such advanced copolymers include a series of amphiphilic NiPAAm-based macromers with dual gelation properties [55,56], calcium ion–sensitive macromers of a similar design with vinylphosphonic acid as comonomer [57], and a series of anhydride group–containing oligomers synthesized with maleic anhydride and a key copolymer [58]. The latter have been used to formulate dual-component hydrogels with gelatin [59], cross-linked gelatin microparticles, as well as tubular conduits [60]. NiPAAm-based copolymers with at least dual functionality have also been synthesized with glycidyl methacrylate as comonomer [61]. The introduction of epoxides allowed for effective cross-linking and dual-component hydrogel engineering with polyamidoamine-based diamines [62]. Syneresis in NiPPAm-based hydrogels could be suppressed and their degradation improved by the integration of dimethyl-γ-butyrolactone acrylate as comonomer with a hydrolysable lactone structure [63,64]. The chemical incorporation of the comonomer monoacryloxyethyl phosphate and subsequent ester formation with glycidyl methacrylate allowed for the introduction of phosphatase degradable linkages in injectable hydrogels for bone regeneration [65,66].

Polyethers

PEG (Fig. 33.2A), often also called poly(ethylene oxide) (PEO), is a nondegradable polyether of the monomer ethylene glycol. Technically, PEG and PEO should not be used as synonyms because PEO is synthesized from the monomer ethylene oxide and typically is terminated by only one hydroxyl group and an initiator fragment [67]. Commonly, PEG is often used to refer to the polymer with a molecular weight less than 30,000–50,000 Da whereas PEO is used for higher molecular weights. PEG is water soluble and solutions of its high–molecular weight form can be categorized as a hydrogel. PEG hydrogels for biomedical applications are typically composed of polymer chains that are cross-linked. These cross-linked networks frequently contain chemical bonds between the PEG chains and the cross-linkable moieties, which are prone to aqueous hydrolysis and therefore are characterized as biodegradable systems. The molecular weight of the PEG chains cross-linked in such hydrogels is below a threshold molecular weight to allow for complete resorption by renal elimination of the individual chains. Consequently, these materials are discussed as biodegradable polymers in the Cross-linked Polyesters section.

Favorable characteristics of PEG and PEO include their high hydrophilicity, bioinertness, and outstanding biocompatibility, which make them candidate biomaterials [68]. PEG and PEO are frequently used as hydrophilic polymeric building blocks in copolymers with more hydrophobic degradable or nondegradable polymers for drug delivery [69], gene delivery, tissue engineering scaffolds, medical devices, and implants. PEG has also been immobilized on polymeric biomaterial surfaces to make them resistant to protein absorption and cell adhesion. These effects are attributed to highly hydrated PEG chains on the polymer surfaces that exhibit steric repulsion based on an osmotic or entropic mechanism [70]. Attempts to benefit from this phenomenon include the design of long-circulating nanoparticles or liposomes [71–74] and PEGylated enzymes or proteins with a prolonged functional residence time in vivo compared with unmodified biomolecules [75,76].

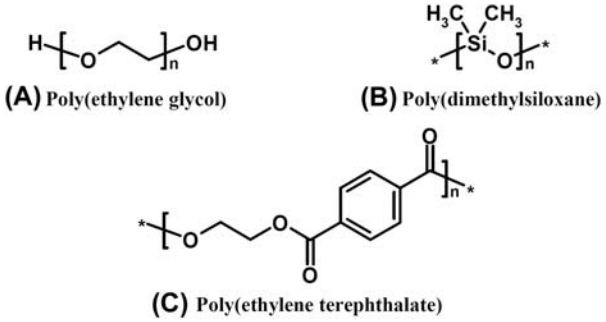

FIGURE 33.2 Chemical structures of nondegradable synthetic polymers (II).

A variety of PEG-containing block copolymers for injectable drug delivery have been developed [77]. The most prominent class is triblock copolymers composed of two hydrophilic PEO blocks and one hydrophobic poly(propylene oxide) (PPO) block, also known as Pluronics or poloxamers. These materials are designed to have phase transition behavior similar to thermogelling PNiPAAm-containing materials (see Poly(*N*-isopropylacrylamide) section). Poloxamers have been intensively investigated for the delivery of drugs and proteins [78]. Because Poloxamers are nondegradable, biodegradable structural analogues have been synthesized. They are described within the next section, Block Copolymers of Polyesters or Polyamides With Poly(ethylene glycol).

Polyglycerols (also called polyglycidols) have gained recognition as an alternative to PEG and PPO. The main distinguishing feature of this polymer is the presence of an additional hydroxyl group on the monomer. As a result, polyglycerols can have a linear structure with a free hydroxyl group per repeat unit or a hyperbranched structure, in which all three hydroxyl groups contribute to the formation of the polyether [79,80]. Linear polyglycerol was found to possess properties similar to PEG with regard to biocompatibility and protein absorption resistance. In addition, the materials exhibit thermoresponsive behavior [81].

Polysiloxanes

Polysiloxanes, or silicones, are a general category of polymers consisting of a silicon-oxygen backbone with organic groups, typically methyl groups, attached to the silicon atoms [82]. Certain organic side groups can be used to link two or more chains. By varying the silicon-oxygen chain length, side groups, and extent of cross-linking, silicones with properties ranging from liquids to hard plastics can be synthesized. Silicone synthesis typically involves the hydrolysis of chlorosilanes into linear or cyclic siloxane oligomers, which are then polymerized into polysiloxanes by polycondensation or polymerization, respectively. The most common polysiloxane is linear poly(dimethylsiloxane) (Fig. 33.2B).

Polysiloxanes, which are characterized by unique material properties combining biocompatibility and biodurability, have found widespread application in health care [82]. The materials' high biodurability is a result of other material properties such as hydrophobicity, low surface tension, and chemical and thermal stability. Silicone surfaces have been found to inhibit blood from clogging for many hours and thus have been used to fabricate silicone-coated needles, syringes, and other blood-collecting instruments. Silicone materials have also been employed as heart valves and components in kidney dialysis, blood oxygenators, and heart bypass machines owing to their hemocompatibility. Silicone elastomers have been used in numerous catheters, shunts, drains, and tubular implants such as artificial urethra. Significant orthopedic applications of silicones are joint implants in hands and feet. The most prominent application of silicones is their extensive use as cosmetic implants in aesthetic and reconstructive plastic surgery. Prosthetic silicone implants are available for the breast, scrotum, chin, nose, cheek, calf, and buttocks. Different silicone materials, including slightly cross-linked silicone gels, are combined to achieve a natural feel. Controversy arose regarding the safety of popular silicone gel-filled breast implants in the early 1990s. These discussions initially involved an increased risk for breast cancer; it then progressed to autoimmune connective tissue disease and evolved to the frequency of local or surgical complications such as rupture, infection, and capsular contracture. To date, no epidemiology study has indicated that the rate of breast cancer has significantly increased in women with silicone breast implants [83]. However, a slight increase in anaplastic large cell lymphoma, a rare cancer of the immune system, is being discussed as connected with breast implants [84]. Studies on autoimmune or connective tissue disease agreed regarding the lack of causal association between breast implants and these diseases [85,86]. A safety concern that was discussed involves the amount of platinum (the part of catalysts used during silicone synthesis) that is released from silicone implants and accumulated in the host organism [87,88]. Other complications, especially implant rupture, are persisting problems; in 1992, the FDA restricted the use of silicone gel-filled implants. Since then, the implants may be used only under certain controlled conditions [89]. In 2016, the FDA approved three types of saline-filled breast implants and five types of silicone gel-filled ones, and required the manufacturers of silicone gel-filled implants to conduct postapproval studies to characterize the safety and effectiveness of their implants.

Polysiloxane gels, which combine the high oxygen permeability of silicone and the comfort and clinical performance of conventional polyacrylate hydrogels, enable the fabrication of soft, gas-permeable contact lenses for extended wear. In contrast to conventional hydrogels, silicone gels render the surface of the lens highly hydrophobic and less "wettable," which frequently results in discomfort and dryness during lens wear. Surface modifications of the silicones or the addition of conventional hydrogels are suitable strategies to compensate for the hydrophobicity.

Overall, polysiloxanes have been increasingly applied in medicine since the 1960s; today, they are one of the most thoroughly tested and important biomaterials.

Other Nondegradable Polymers

Poly(ethylene Terephthalate)

Poly(ethylene terephthalate) (Fig. 33.2C), a linear polyester synthesized by polycondensation of terephthalic acid and ethylene glycol, is typically processed into fiber meshes. These meshes are applied as vascular grafts [27] or used to reinforce prostheses.

Hydrolytically Stable Polyurethanes

PUs are a heterogeneous class of polymers that consist of organic units joined by urethane links (Fig. 33.3). Generally, PUs can be synthesized from a bischloroformate and a diamine or by reacting a diisocyanate with a dihydroxy component. PUs used in biomedical applications typically have a segmented structure that results in useful physicochemical properties [90]. Such segmented PUs or PU copolymers are elastomers composed of alternating polydispersed "soft" and "hard" segments. These two segments are thermodynamically incompatible and phase-segregate, resulting in discrete, crystalline domains of the associated "hard" segments surrounded by a continuous, amorphous phase of "soft" segments. The segregated domains are stabilized by interchain hydrogen bonds and are responsible for the materials' mechanical properties [91]. Segmented PUs are synthesized in a two-step process that provides control over polymer architecture (Fig. 33.3A). The first step involves the synthesis of an isocyanate-terminated prepolymer from a diisocyanate (D in Fig. 33.3) and a hydroxyl group terminated polyether or polyester (P in Fig. 33.3). The prepolymer and excess diisocyanate are then reacted with a hydroxy or amine group-terminated chain extender (C in Fig. 33.3) to generate the final PU (Fig. 33.3A).

A chain extender terminated with hydroxy groups yields segmented PUs, whereas a diamine extender yields PU-urea (Fig. 33.3B). The "hard" segment of the PU copolymer is composed of the diisocyanate and the chain extender, whereas the "soft" segment contains the polymeric segment introduced during the first step. The extent of phase separation depends on the molecular weights, chemistry, and relative percentages of the building blocks [92].

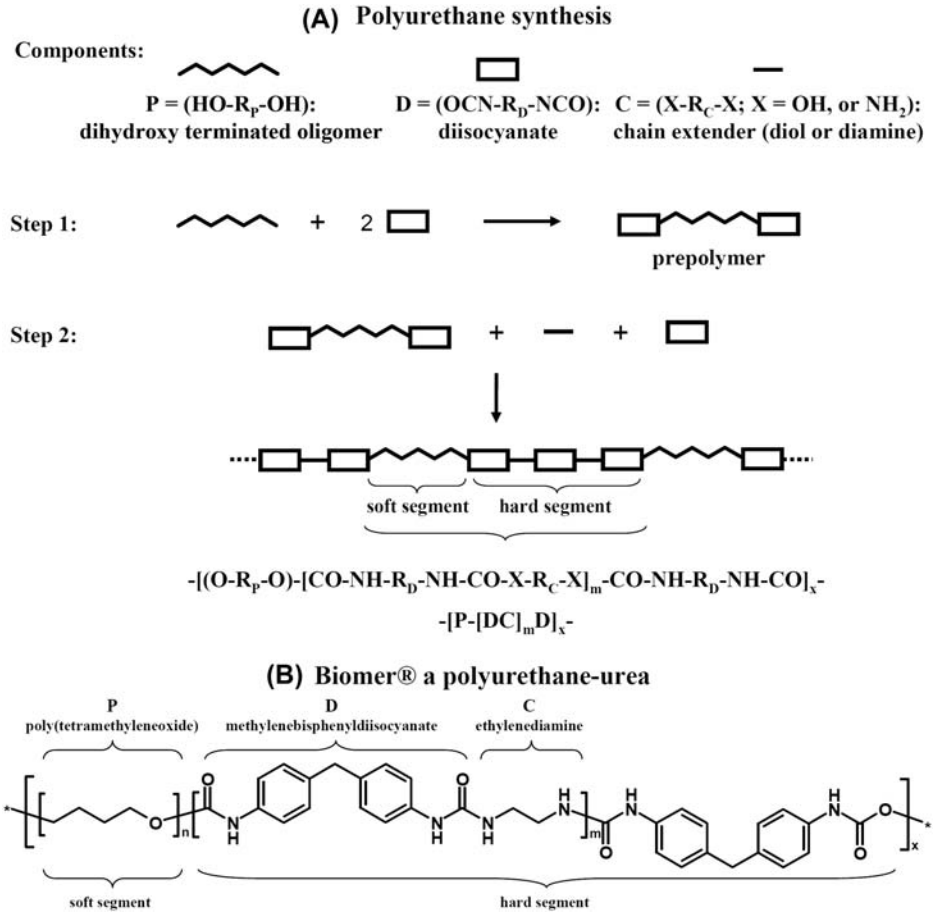

FIGURE 33.3 General synthesis scheme (A) and an example structure (B) for polyurethanes.

After almost 50 years of use in biomedical applications, PUs remain one of the most popular groups of biomaterials for the fabrication of medical devices. Their popularity results from a wide range of versatility with regard to tailoring their physicochemical and mechanical properties, blood and tissue compatibility, and degradative properties by altering block copolymer composition. Biomedical PUs are used in numerous medical devices such as breast implants, catheters, vascular and aortic grafts, pacemaker leads, artificial heart valves, and artificial hearts; they perform well in a variety of in vivo applications. PUs often have better blood and tissue compatibilities compare with numerous other synthetic polymers. The efficient removal of impurities from the polymer synthesis, such as catalyst residues and low−molecular weight oligomers, has critically determined PU biocompatibility [93].

Traditional PUs such as Biomer (in which the dihydroxy-terminated oligomer (P) is polytetramethylenen oxide, the diisocyanate (D) is methylene bisphenylenediisocyante and ethylenediamine is the chain extender (C)) (Fig. 33.2D), were materials of first choice. However, the assumption of polyetherurethane nondegradability had to be revised in response to the well-documented failures of pacemaker leads and breast implant coatings containing PUs in the late 1980s. Although PUs can be designed to be stable against hydrolysis, these materials have been shown to degrade in the biological environment by mechanisms including oxidation and enzyme and cell-mediated degradation [92,94,95]. Oxidation of PUs can be initiated by peroxides, free radicals, and enzymes. Metal-catalyzed oxidation was found to be most frequently associated with pacemaker lead failure. Another important oxidation-driven problem with long-term PU implants is environmental stress cracking. PU surfaces become coated with a protein layer that enhances the adhesion of macrophages. The macrophages, activated by proteins of the complement family, release oxidative factors that accelerate the degradation of the polymer [96].

Chemical design criteria have been identified for biostable PUs. To increase the degree of interchain hydrogen bonding, on which biostability partially depends, low−molecular weight oligomeric diols (P) are preferred as building blocks. To avoid oligomer hydrolysis, oligoethers are favored over oligoesters. Aromatic diisocyanates (D) have been found to yield more biostable PUs than aliphatic diisocyanates. The use of a diamine chain extender (C) instead of a dihydroxy-terminated one typically results in stronger PU-urea, but polymer fabrication is often hampered because of solubility problems. The use of soft segment building blocks with high crystallinity, such as polycaprolactone, or silicone-based oligomers is also assumed to improve polymer biostability [92].

PUs can be surface modified to reduce the risk of thrombosis or improve interactions with cells and tissues. Different strategies, including adsorption, covalent grafting, and the use of self-assembled monolayers, have been applied to distribute proteins such as fibronectin, or adhesion peptides, which contain the integrin-binding peptide motif RGD, across the PU surface [92,97].

BIODEGRADABLE SYNTHETIC POLYMERS FOR REGENERATIVE MEDICINE

Biodegradable synthetic polymers offer a number of advantages over nondegradable materials for applications in regenerative medicine. Like all synthetic polymers, they can be synthesized at reproducible quality and purity and fabricated into various shapes with desired bulk and surface properties. Specific advantages include the ability to tailor mechanical properties and degradation kinetics to suit various applications. Clinical applications for biodegradable synthetic polymers are manifold and traditionally include resorbable sutures, drug delivery systems, and orthopedic fixation devices such as pins, rods, and screws [98]. Synthetic biodegradables were widely explored as artificial matrices for tissue engineering applications [99−103]. For such applications, the mechanical properties of the scaffolds, which are determined by the constitutive polymer, should functionally mimic the properties of the tissue to be regenerated. Ultimately, the polymeric support is designed to degrade while transplanted or invading cells proliferate, lay down ECM, and form coherent tissue that ideally is functionally, histologically, and mechanically indistinguishable from the surrounding tissue. To engineer scaffolds suitable for different applications, a wide variety of biodegradable polymers are required ranging from pliable, elastic materials for soft tissue regeneration to stiff materials that can be used in load-bearing tissues such as bone. In addition to the mechanical properties, the degradation kinetics of polymer and ultimately scaffold also have to be tailored to suit various applications.

The major classes of synthetic, biodegradable polymers are briefly reviewed and their potential in regenerative medicine is discussed subsequently.

Polyesters

Polyesters have been attractive for biomedical applications because of their ease of degradation by the primarily nonenzymatic hydrolysis of ester linkages along the backbone. In addition, degradation products can be

resorbed through the metabolic pathways in most cases, and there is the potential to tailor the structure to alter degradation rates [104].

A vast majority of biodegradable polymers studied belong to the polyester family [105]. Polyester fibers, which also became popular in the textile industry, were used as resorbable sutures [106]. Promising observations regarding the biocompatibility of the materials led to applications in drug delivery, orthopedic implants, and tissue engineering scaffolds, particularly for orthopedic applications [98,102,107–110].

Polyesters of α-Hydroxy Acids

The family of polyesters can be subdivided according to the structure of the monomers. In poly(α-hydroxy acids), each monomer has two functionalities, a carboxylic acid and a hydroxyl group, located at the carbon atom next to the carboxylic acid (α-position), that form ester bonds. Poly(α-hydroxy acids) are linear thermoplastic elastomers that typically are synthesized by ROP of cyclic dimers of the building blocks [111,112]. Poly(lactic acid) (PLA) (Fig. 33.4A), poly(glycolic acid) (PGA) (Fig. 33.4B), and a range of their copolymers, including poly(lactic-*co*-glycolic acid) (PLGA) (Fig. 33.4C) are prominent representatives not only of biodegradable polyesters but of biodegradables in general. The cyclic dimers that are polymerized during PLA and PGA synthesis are called lactide and glycolide, respectively. Therefore, the polymers are often named polylactides or polyglycolides. For reasons of consistency with the general term poly(α-hydroxy acids), the terms PLA and PGA will be used here. Poly(α-hydroxy acids) have a long history of use as synthetic biodegradable materials in a number of clinical applications. Initially, resorbable sutures were made from these materials [113]. Later, poly(α-hydroxy acids) were the basis for controlled release systems for drugs, proteins and vaccines [114–118], and orthopedic fixation devices [119]. Langer and coworkers pioneered the development of these polymers in the form of porous scaffolds for tissue engineering [120].

Because of the chiral nature of lactic acid, several forms of PLA exist: poly(L-lactic acid) (P_LLA), for example, is synthesized from L-lactide. The polymerization of racemic lactide leads to poly(D,L-lactic acid) ($P_{D,L}LA$), which is an amorphous polymer. P_LLA, in contrast, is a semicrystalline polymer with a crystallinity of around 37%. P_LLA is characterized by a glass transition temperature between 50°C and 80°C and a melting temperature between 173°C and 178°C. Amorphous $P_{D,L}LA$ is typically used in drug delivery applications, whereas semicrystalline P_LLA is preferred in applications in which high mechanical strength and toughness are required, e.g., for sutures and orthopedic devices. PGA is also a semicrystalline polymer with a higher crystallinity of 46–52%. Thermal characteristics of PGA are glass transition and melting temperatures of 36°C and 225°C, respectively. Because of its high crystallinity, unlike PLA, PGA is not soluble in most organic solvents; the exceptions are highly fluorinated organic solvents such as hexafluoroisopropanol. Consequently, common processing techniques for PGA include melt extrusion, injection, and compression molding.

PLA, PGA, and PLGA undergo homogeneous erosion via ester linkage hydrolysis into the degradation products lactic acid and glycolic acid, which are both natural metabolites that are fully metabolized and excreted as carbon dioxide and water. Degradation of poly(α-hydroxy acid)s showed characteristics typical of bulk erosion. Bulk erosion occurs when water penetrates the entire structure and the device degrades simultaneously [121]. During the initial stages of degradation, almost no mass loss can be detected. Analysis of the average molecular weight of the polymer bulk over the same period, however, reveals a steady decrease in molecular weight. Once the polymer chains throughout the bulk are degraded below a certain threshold, the water-soluble degradation products are washed out and the system collapses, accompanied by significant mass loss. Because of its well-accessible ester group, PGA degrades rapidly in aqueous media. PGA sutures typically lose their mechanical strength over 2–4 weeks postoperatively [122]. To adapt these properties to a wider range of applications, copolymers with more hydrophobic PLA were synthesized and investigated. The two main series are those of P_LLGA (Fig. 33.4C) and $P_{D,L}LGA$. It was shown that the range of compositions from 25% to 70% glycolic acid (GA) for L-lactic acid (L-LA)/GA and from 0% to 70% GA for D,L-LA/GA are amorphous [104,105,123–126]. For the P_LLGA copolymers, the rate of hydrolysis was slower at either extreme of the copolymer composition range. It is generally accepted that intermediate PLGA copolymers have a shorter half-life in vivo than either homopolymer. Besides the polymer composition, the rate of degradation is affected by factors such as the configurational structure, copolymer ratio, crystallinity, molecular weight, morphology, stresses, the amount of residual monomer, bulk porosity, and the site of implantation [104].

Multiple in vitro and in vivo studies that were conducted on the biocompatibility of PLA, PLGA, and PGA have generally revealed satisfying results [127]. Consequently, PLA, PLGA copolymers, and PGA are among the few biodegradable polymers with FDA approval for human clinical use.

Concerns with poly(α-hydroxy esters) typically focus on the accumulation of acidic degradation products within the polymer bulk that can have detrimental effects on encapsulated drugs in delivery applications [128–130] or can cause late noninfectious inflammatory responses when released in a sudden burst upon structure breakdown

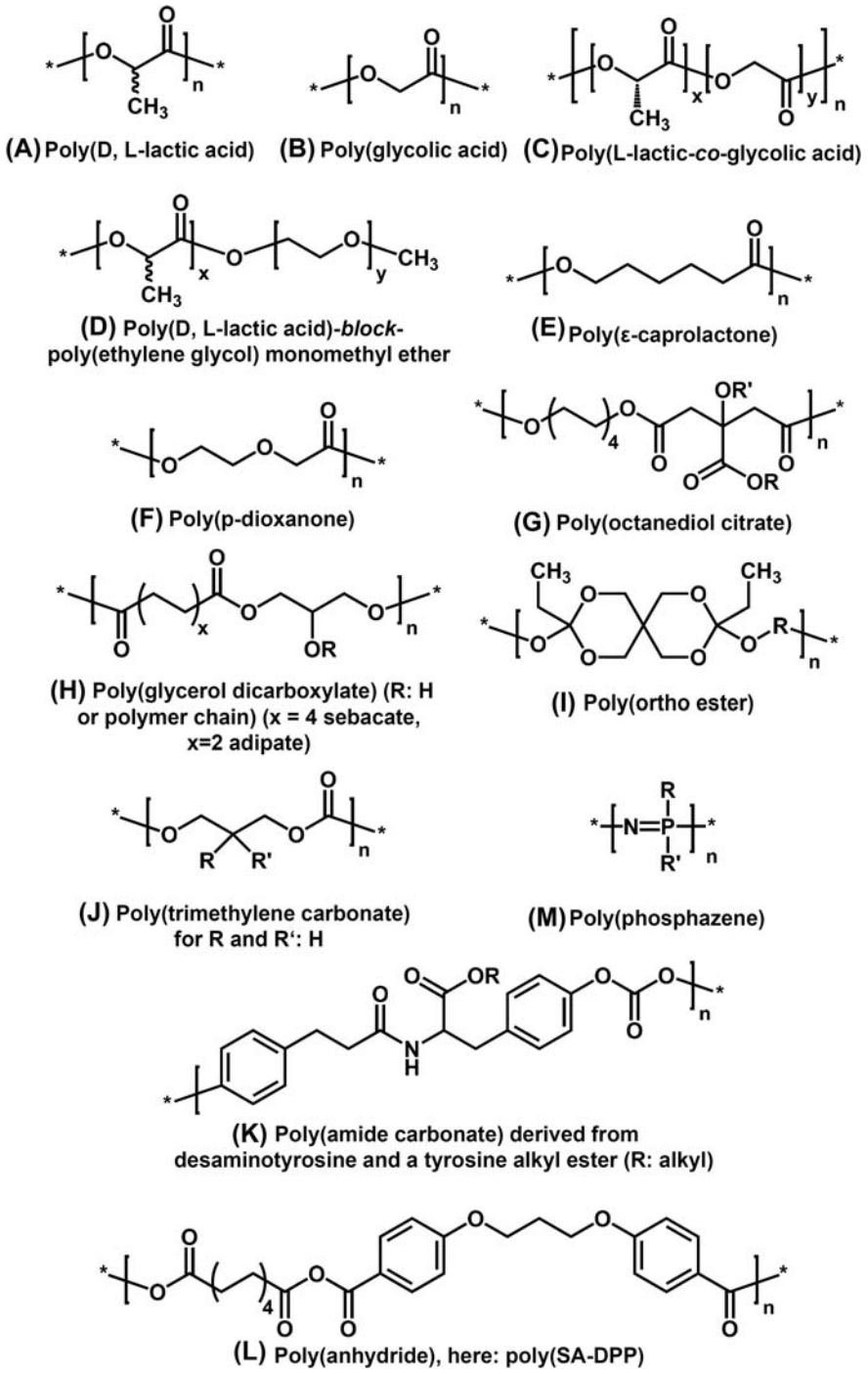

FIGURE 33.4 Chemical structures of biodegradable synthetic polymers.

[131–133]. This adverse reaction can occur weeks and months postoperatively and might need surgical drainage. This is a major concern in orthopedic applications, in which implants of considerable size would be required and which may result in the release of degradation products with high local acid concentrations. Inflammatory response to poly(α-hydroxy acids) were also found to be triggered by the release of small particles during degradation that were phagocytosed by macrophages and multinucleated giant cells [117,134]. In general, implant size as well as surface properties appear to be critical factors with regard to biocompatibility. Fewer concerns seem to exist regarding the application of poly(α-hydroxy acids) in soft tissues compared with hard tissue applications [127]. Injectable P_LLA has been employed for skin volume restoration by using its stimulating effect on collagen production [135].

Poly(α-hydroxy acids) were the materials of choice when one of the key concepts of tissue engineering, the de novo engineering of tissue by combining isolated cells and three-dimensional macroporous cell carriers in vitro, was first realized and developed [120,136,137]. Polymers based on lactic acid and GA are still popular scaffold materials, especially for orthopedic applications such as bone, cartilage, and meniscus, as outlined in several reviews [101,138–140]. Limitations of this class of materials include insufficient mechanical properties with regard to load-bearing applications [110] and inflammatory or cytotoxic events caused by the accumulation of acidic products during degradation.

To cover a broader range of mechanical and physicochemical properties, such as water absorption, polymer degradation, and polymer–drug interactions, block copolymers containing PLA and hydrophilic PEO or PEG were synthesized for drug delivery applications [141,142]. Solid particulate systems from these block copolymers were almost invisible to the immune system owing to the hydrophilic PEG chains that swell on the surface [71,143] (see Polyethers section) (Fig. 33.4D). The stealthiness of such surfaces is mainly caused by the suppression of protein adsorption, which also inhibits cell adhesion. Investigations into cell adhesion to PEG–PLA diblock copolymer surfaces revealed that cell adhesion can be controlled and cell differentiation can be modulated by the PEG content [144]. With the objective of specifically controlling cell–polymer interactions, PEG–PLA copolymers were further developed to allow for the covalent attachment of signaling molecules [145,146]. Because these polymers were insoluble in water, they could be processed into macroporous scaffolds for tissue engineering applications [147]. Furthermore, diblock and triblock copolymers of PLA with PEG were investigated as microscale and nanoscale vehicles for drug delivery [148,149], as well as for tissue engineering applications [150].

Another direction in which to broaden the range of mechanical and chemical properties of PLA centers on the use of α-hydroxy acids that possess longer alkyl or aryl side chains [151] or feature a side chain with an additional double or triple bond or a functional group [152]. An important effect of such an incorporation is the modulation of the glass transition temperature of the resulting polymer [153]. For example, a viscous copolymer of lactic acid and 2-hydroxy-octanoic acid was investigated as a delayed intravitreal release system for vasodilatory substances or as an excipient for sustained-release drug formulations [154–156].

Polyesters of Lactones

The most prominent and thoroughly investigated polylactone is poly(ε-caprolactone) (PCL) (Fig. 33.4E), an aliphatic, semicrystalline polyester with an interestingly low glass transition temperature (−60°C) and melting temperature (59-64°C) [105,157]. PCL is considered to be biocompatible [158]. PCL is prepared by the ROP of the cyclic monomer ε-caprolactone and is compatible with a range of other polymers. Catalysts such as stannous octoate are used to catalyze the polymerization and low–molecular weight alcohols can be used as the initiator and to control the molecular weight of the polymer [159]. ε-Caprolactone can be copolymerized with numerous other monomers. Copolymers with PLA and PEG are probably the most noteworthy and have been investigated extensively [160–163]. PCL degrades at a much slower rate than PLA and therefore is most suitable for the development of long-term implantable drug delivery systems. These copolymers of caprolactone with lactide were synthesized to accelerate degradation rates [105]. Tubular, highly permeable poly(L-lactide-*co*-ε-caprolactone) guides were found to be suitable for the regeneration and functional reinnervation of large gaps in injured nerves [164]. Although this study focused on tissue regeneration, the application of PCL in drug delivery devices is still far more common [165]. With the increasing popularity of electrospinning, a laboratory-scale technique that allows for the fabrication of nonwoven meshes composed of nanofibers and/or microfibers [166], PCL might find its way into cell-based therapies because slowly degrading polymers are preferred for this technique to ensure sufficient stability of the fibers [167].

Poly(p-dioxanone) (Fig. 33.4F), another polylactone, and its copolymers with lactide, glycolide, and/or trimethylene carbonate, are synthesized by catalyzed ROP and have been used in a number of clinical applications ranging from suture materials to bone fixation devices [168,169]. The use of analogues of p-dioxanone bearing methyl or more complex groups for homopolymerization or copolymerization allows for the fine-tuning of degradation properties of the polymer [170].

Another class of polymer that is accessible via the polymerization of lactones is poly(hydroxy-alkanoates) (PHAs), which are composed of hydroxy acids with the hydroxyl group located at least in the β-position relative to the carboxylic acid group [171]. PHAs are mostly produced biosynthetically; the resulting polymer is perfectly isotactic and highly crystalline. Synthesis via ROP of lactones, on the other hand, enables the production of syndiotactic and atactic PHAs with altered physicochemical and mechanical properties [172].

Polyesters of Polyols and Carboxylic Acids

In addition to using monomers bearing a hydroxyl and a carboxyl group on the same molecule, elastomeric polyester networks have been synthesized by combining two kinds of building blocks, one bearing two or more hydroxyl groups (a polyol), such as glycerol [173], and one bearing two or three carboxyl groups (a dicarboxylic acid or tricarboxylic acid), such as citric acid [174]. Polymers are synthesized from the two building blocks by polycondensation either by organometallic, metal-oxide [175], enzyme-based catalysis [176] or without using exogenous catalysts [177,178], depending on the employed building blocks.

Poly(diol citrates) are synthesized from citric acid and various low− or high−molecular weight diols [174]. Poly(1,8-octanediol-*co*-citrate) (POC) (Fig. 33.4G; R, R′: −H), one of the first poly(diol citrates), demonstrated mechanical properties such as tensile strength, Young's modulus, and elongation at break that justify applications in ligament reconstruction and vascular engineering [178]. Variations in chemical composition, especially diol chemistry, allowed for the synthesis of a variety of biodegradable elastomers covering a range of mechanical and degradative properties [179]. With regard to vascular tissue engineering, POC showed good hemocompatibility and exhibited decreased platelet adhesion and clotting relative to P_LLGA and expanded poly(tetrafluoro ethylene) [180]. Endothelial cell attachment and differentiation were supported with no modification of the surface. To improve the mechanical properties of the POC elastomer, unsaturated acrylate and fumarate diols were added during the condensation reaction and moieties for secondary cross-linking were introduced [181]. Further modification of the employed diols and functionalization of the pendant carboxyl and hydroxyl groups of citric acid led to citrate-based biomaterials with potential applications ranging from regeneration of hard and soft tissue to drug delivery, bioadhesive, and imaging functions [174].

Poly(glycerol sebacate) (Fig. 33.4H; $x = 4$) is an elastomeric polymer network made from the triol glycerol and sebacic acid (decanedioic acid) [182]. It is developed for use in the regeneration of soft tissues such as cardiac [183], vascular, retinal, and neural tissue. A similar polymer, poly(glycerol adipate) (Fig. 33.4H; $x = 2$), made from glycerol and adipic acid (hexanedioic acid), is being examined for use as a nanoparticulate drug delivery system after esterification with fatty acids [184].

Linear polymers of this type are synthesized from a diol and a dicarboxylic acid [185]. An example is poly(butylene succinate), which is synthesized from 1,4-butanediole and succinic acid [186] and which has been evaluated for use in the regeneration of cartilage [187] and bone tissue [188].

Polyorthoesters

Polyorthoesters (POEs) (Fig. 33.4I) were developed by Alza Corporation and SRI International in 1970 in the search for a new biodegradable polymer for drug delivery applications [189]. Since then, polymer synthesis has improved. POEs are synthesized by condensation or addition reactions typically involving dialcohols and monomeric orthoesters or diketene acetals, respectively. The use of triethylene glycol as the diol component produced predominantly hydrophilic polymers, whereas hydrophobic materials could be obtained by using 1,10-decanediol. Orthoester is a functional group containing three alkoxy groups attached to one carbon atom. In POEs, two of the three alkoxy groups are typically part of a cyclic acetal (Fig. 33.4G).

POEs were synthesized that degrade by surface erosion, which is characterized by a constant decrease in bulk mass while the polymer molecular weight within the polymer bulk is preserved [190]. It is known that materials built from functional groups with short hydrolysis half-lives and low water diffusivity tend to be surface eroding. Polymers that exhibit surface erosion can be used to fabricate drug delivery systems that release loaded drugs at a constant rate at a high aspect to volume ratio (e.g., as for wafers).

The addition of lactide segments to the POE structure resulted in self-catalyzed erosion and allowed for fine-tunable degradation times ranging from weeks to months [191]. POEs provide the material platform for a variety of drug delivery applications including the treatment of postsurgical pain, osteoarthritis and ophthalmic diseases, and the delivery of proteins and DNA. Block copolymers of POE and PEG have been prepared and their use as drug delivery matrices or as colloidal structures for tumor targeting are being explored [189].

Initial biocompatibility studies revealed that POEs provoked little inflammation and were largely absorbed by 4 weeks. In contrast, $P_{D,L}LA$ degraded more slowly and provoked chronic inflammation with multinuclear giant cells, macrophages with engulfed material, and proliferating fibroblasts within the same model. Ossicles with bone marrow had formed in the implants of POE combined with demineralized bone. In PLA/demineralized bone implants, bone formation was inhibited [192,193].

Polycarbonates

Polycarbonates have become interesting biomaterials because of their excellent mechanical strength and good processability. The degradation of most polycarbonates is controlled by hydrolysis of the carbonate group, which yields two alcohols and carbon dioxide, which alleviates the problem of acid bursting seen in polyesters [104,194]. The structural variation in the pendant side groups allows polymers to be prepared with different mechanical properties, degradation rates, and cellular responses.

The most prototypical polycarbonate is poly(trimethylene carbonate) (Fig. 33.4J), generated by ROP of the monomer 1,3-dioxan-2-one (trimethylene carbonate [TMC]). By using substituted analogues of this monomer, it is possible to introduce additional functionalities into the material [195]. Altered material properties resulting from these functionalities widen the prospect for the use of polycarbonates in tissue and bone regeneration [196,197] as well as in drug delivery [198,199] and as polymer-based antibiotics [200,201].

Because TMC-based polycarbonates degrade extremely slowly under physiological conditions [195], polyiminocarbonates [202] and tyrosine-based polycarbonates [203] (Fig. 33.4K) have been engineered to yield biodegradable polymers with good mechanical strength [204] for use in drug delivery and orthopedic applications. Tyrosine-based polycarbonates that contain a pendant ethyl ester group have been shown to be osteoconductive and to possess mechanical properties sufficient for load-bearing bone fixation. Long-term (48-week) in vivo degradation kinetics and host bone response to tyrosine-derived polycarbonates were investigated using a canine bone chamber model [205]. Histological sections revealed intimate contact between bone and the polymer. It was concluded that from a degradation-biocompatibility perspective, the tyrosine-derived polycarbonates appear to be comparable to PLA, if not superior, in this model. The incorporation of desaminotyrosyltyrosine units without a pendant ester and low–molecular weight PEG allows for the development of tyrosine-based polycarbonates with faster degradation without affecting the osteoconductive properties [206], which are further improved upon by combining the polymer scaffolds with calcium phosphate minerals and bone morphogenetic protein 2 (BMP-2) [207]. Variations in the composition of these terpolymers can be used to engineer ultrafast degrading and resorbing polymers that may be suitable for the coating of implants in brain tissue to minimalize adverse effects [208].

Block Copolymers of Polyesters or Polyamides With Poly(ethylene Glycol)

Amphiphilic block copolymers of biodegradable polymers with PEG have become popular materials for injectable drug delivery applications [78]. Inspired by the thermoresponsive behavior observed for nondegradable A-B-A–type triblock copolymers composed of hydrophilic PEO (block A) and hydrophobic PPO (block B), polymer development focused on synthesizing biodegradable analogs of these poloxamers (or Pluronics) that were water soluble at ambient temperature and formed stable hydrogels at body temperature. Biodegradable block copolymers were synthesized by substituting the hydrophobic PPO block with a biodegradable polymer block such as PLA or PCL [69,77,209].

Biodegradable, physically cross-linkable block copolymers of inverse structure (that is, B-A-B triblock copolymers with two biodegradable hydrophobic polymer blocks [block B] and a hydrophilic PEO block) were also investigated as protein delivery systems [148,210].

Polyurethanes

PUs represent a major class of synthetic elastomers that have excellent mechanical properties and good biocompatibility. PUs have been evaluated for a variety of medical devices and implants, particularly for long-term implants.

Knowledge gained about the mechanisms of PU biodegradation in response to implant failures throughout the 1990s has been translated to form a new class of bioresorbable materials [95]. Research has employed the flexible chemistry and diverse mechanical properties of PUs to design degradable polymers for a variety of regenerative applications. Segmented PUs with varied molecular structure have been synthesized to control the rates of hydrolysis [95,211]. To obtain biodegradable, segmented PUs, significant changes were required to the structural components historically used for their synthesis. Traditional aromatic diisocyanates (D; compare with Fig. 33.3) can yield toxic or carcinogenic degradation products when they are part of a degradable PU; therefore, linear diisocyanates are preferred, such as lysine-diisocyanate, which yields the nontoxic degradation product lysine. The soft segment, which is typically composed of an oligomeric diol (P; compare with Fig. 33.3), is typically the block of the PU used to modify the degradation rate. Biodegradable PUs have been synthesized with a variety of soft segments including PEO, degradable polyesters such as PLA, PGA, or PCL, and their combinations. Other strategies focus on the copolymers' hard segments. PUs were synthesized that contain enzyme-sensitive linkages introduced with

the chain extender (C; compare with Fig. 33.3). For example, the use of a phenylalanine diester chain extender yielded a PU that showed susceptibility to enzyme-mediated degradation upon exposure to chymotrypsin and trypsin.

Saad et al. investigated cell and tissue interactions with a series of degradable polyesterurethanes. In vivo investigations showed that all test polymers exhibited favorable tissue compatibility and degraded significantly over 1 year [212]. Polyurethane-urea matrices were shown to allow vascularization and tissue infiltration in vivo [213]. The flexible chemistry and diverse mechanical properties of PU materials allowed researchers to design degradable polymers to regenerate diverse tissues including neurons, vasculature, smooth muscle, cartilage, and bone [27,95,214].

To accelerate the degradation behavior of polyesterurethanes, polyester segments in the polymer backbone were partially substituted with polycarbonate, yielding poly(ester carbonate)urethane-ureas [215]. Flexible, low-moduli biodegradable PU, or poly(ether carbonate urethane)-ureas, were synthesized with polyether-based domains containing biodegradable sections as soft segments [216]. Examples of such soft segments include poly(trimethylene carbonate) (PTMC)-based triblock copolymers such as PTMC−PEO−PTMC and thermoresponsive pentablock copolymers such as PTMC−PEO−PPO−PEO−PTMC. For controlling the response of a biological system to materials, surface and bulk modifications are a common strategy. To this end, a series of biodegradable polyesterurethane-urea elastomers with variable amino content were developed. Via the amine groups, carboxylated phosphorylcholine was conjugated to polymer for bulk functionalization [217]. This modification significantly reduced platelet adhesion to the material and inhibited rat vascular smooth muscle cell proliferation. Such materials may find use as coatings in cardiovascular devices or as scaffolds for cardiovascular tissue regeneration. Enzyme-degradable PU was synthesized by using a collagenase-sensitive peptide as a chain extender [218].

Amino Acid−Derived Polymers, Poly(amino Acids), and Peptides

Amino acids are an interesting building block for polymers because of the biocompatibility of the degradation products and the degradability of the amide or ester bonds by which amino acids are typically polymerized or integrated in copolymers. Early studies on pure poly(amino acids)s revealed significant concerns regarding the materials' immunogenicity and mechanical properties [219]. To improve those unfavorable properties, amino acids have been used as monomeric building blocks in polymers that have a backbone structure different from that of natural peptides. Based on polymer structure and chemistry, four major groups have been used to classify such "nonpeptide amino acid−based polymers": (1) synthetic polymers with amino acid side chains, (2) copolymers of natural amino acids and non−amino acid monomers, (3) block copolymers containing peptide or poly(amino acid) blocks, and (4) pseudopoly(amino acids) such as α-peptoids [220].

As in tyrosine-derived polycarbonates (discussed in the Polycarbonates section), L-tyrosine is the predominantly employed amino acid in the synthesis of tyrosine-derived polyarylates and polyesters. These copolymers exhibit excellent engineering properties and polymer systems can be designed whose members have exceptional strength (polycarbonates), flexibility and elastomeric behavior (polyarylates), or water solubility and self-assembly properties (copolymers with PEG). Poly(DTE carbonate) (Fig. 33.4K; R: $-CH_2CH_3$) exhibits a high degree of tissue compatibility and is being evaluated by the FDA for possible clinical use [219].

A combinatorial library of degradable tyrosine-derived polyarylates was synthesized by copolymerizing 14 different tyrosine-derived diphenols and eight different aliphatic diacids in all possible combinations, resulting in 112 distinct polymers [221]. Significant differences were observed in the mechanical properties of the polymers and fibroblast proliferation assays with these materials. This illustrates that such combinatorial approaches provide a library of related polymers that encompasses a broad range of properties and permits the systematic study of material-dependent biological responses in order to choose a suitable material for a specific application.

Another amino acid that has received interest as a component in synthetic polymers for biomedical purposes is L-lysine. Lysine has been investigated as a component in urethane/urea esters, both as a linear block with a free carboxyl group for functionalization [222] and as a three-armed branching point in which the carboxyl group bears an ester with another amino functionality. Degradation studies of the latter material pointed to oxidative processes having an important role in the degradation of such a material in vivo [223]. Lysine was also employed in block copolymers with PEG and PLA that showed no cytotoxic effect [224], in an allylamine-initiated homopolymer copolymerized with PEG-diacrylate as a hydrogel for nerve regeneration [225] or in a genipin-cross-linked poly(L-lysine) hydrogel for drug delivery, and as a cell culture substrate [226].

In a more general context, the polymerization of amino acids with side chain functionalities yields polymer chains with pendent functional groups that can be used for further functionalization. In addition to lysine and tyrosine, other amino acids have been selected for their side chain functionalities [227], including glutamic acid [228] and

L- dihydroxyphenylalanine [229] to influence calcium phosphate mineralization and L-arginine for the synthesis of urethane/urea polymers as biodegradable gene therapy vector [230].

The most common route to generate homopolymers of amino acids or a poly(amino acid) segment in block copolymers uses the ROP of N-carboxy-anhydrides (NCAs) [231,232]. This process yields polymers that are composed of a single amino acid or a random sequence of amino acids when multiple NCAs are reacted simultaneously. Block copolymers can be obtained when NCAs of different amino acids are polymerized in a stepwise manner. An important issue of long chains of these synthetic poly(amino acids) is high immunogenicity [219]. In contrast, solid-phase peptide synthesis, pioneered by Merrifield, and genetic engineering allow for the automated and highly efficient synthesis of peptides of a predefined sequence. Synthetic peptides have become an important polymer class for biomedical applications. Specifically, peptides and peptide-amphiphiles that undergo self-assembly—driven in situ gelation in response to temperature, pH, or chemical stimuli are interesting because these materials can be minimally invasively implanted starting from aqueous solutions [233–235].

Genetically engineered elastin-like polypeptides, which are composed of a pentapeptide repeat and undergo inverse temperature phase transition, have been used to encapsulate chondrocytes. Cell culture studies showed that cartilaginous tissue formation was supported, characterized by the biosynthesis of sulfated glycosaminoglycans and collagen [236].

Self-assembled peptide-amphiphiles form hydrogels composed of nanofibers resembling the native ECM components, with the amphiphiles composed of a peptide with hydrophilic and hydrophobic domains, a hydrophilic peptide attached to a hydrophobic lipid chains, or copolymers composed of the peptide and a hydrophobic component [237]. These hydrogels have been demonstrated to be cytocompatible in cell encapsulation studies [238]. Peptide nanostructures designed through self-assembly strategies and supramolecular chemistry have the potential to combine bioactivity with biocompatibility [239]. In addition, such structures can be used to deliver proteins, nucleic acids, drugs, and cells.

Peptide-amphiphile nanofibers were shown to promote the in vitro proliferation and osteogenic differentiation of marrow stromal cells [240]. For dental tissue engineering, dental stem cells were encapsulated in peptide-amphiphile hydrogels containing adhesion peptides and enzyme-cleavable sites. The cells proliferated and differentiated within the gels and remodeled the matrices [241].

Polyanhydrides

Drug delivery technologies rely on engineered polymers that degrade in a well-controllable and adjustable fashion [22]. An increased understanding of erosion mechanisms led to a demand for synthetic polymers that contain a hydrolytically labile backbone while limiting water diffusion within the polymer bulk significantly to confine erosion to the polymer—water interface. Such surface-eroding polymers allow for the fabrication of drug delivery devices that erode at constant velocity at any time during erosion, thus releasing incorporated drugs at constant rates [242]. Polyanhydrides were engineered following this paradigm by selecting the anhydride linkage, one of the least hydrolytically stable chemical bonds available, to connect the building hydrophobic monomers.

Polyanhydrides (Fig. 33.4L) have been synthesized by various techniques, including melt condensation, ROP, interfacial condensation, dehydrochlorination, and dehydrative coupling agents [243]. Solution polymerization traditionally yielded low—molecular weight polymers. Different dicarboxylic acid monomers have been polymerized to yield polyanhydrides with various physicochemical properties. Examples are linear, aromatic, or fatty acid—based dicarboxylic acid monomers, and fatty acid—terminated polyanhydrides. Polyanhydrides made from linear sebacic acid (SA) and aromatic 1,3-*bis*(*p*-carboxyphenoxy)propane (CPP) (Fig. 33.4L) have been engineered to deliver carmustine (BNCU), an anticancer drug, to sites in the brain after primary resection of a malignant glioma [244]. Poly(SA-CPP) hydrolyzes into nontoxic degradation products and local chemotherapy with BCNU wafers was shown to be tolerated and to offer a survival benefit to patients with newly diagnosed malignant glioma, although this therapy revealed potentially problematic side effects [245,246].

The chemical composition of a polyanhydride can be used to custom-design its degradation properties. Whereas polyanhydrides from linear monomers such as poly(SA) degrade within a few days, polymerized aromatic dicarboxylic acids such as poly(1,6-*bis*[*p*-carboxyphenoxy]hexane) degrade much more slowly (up to a year) [247,248]. Combined with their unique degradation and erosion properties, the structural versatility of polyanhydrides make them precious materials for numerous medical, biomedical, and pharmaceutical applications in which degradable polymers that allow for perfect erosion control are needed [242]. With regard to tissue engineering applications, polyanhydrides have also been interesting polymers owing to their degradative properties and their good biocompatibility [249]. The use of polyanhydrides in load-bearing orthopedic applications, however, is restricted because of their limited mechanical properties. Poly(anhydrides-*co*-imides), which were developed to combine

the good mechanical properties of polyimides with the degradative properties of polyanhydrides, were shown to have compressive strengths comparable to human bone [250] and displayed good osteocompatibility [251].

Photopolymerizable polyanhydrides have been synthesized with the objective of combining high strength, controlled degradation, and minimal invasive techniques for orthopedic applications and were shown to be osteocompatible [252]. Depending on the chemical composition, these materials reached compressive and tensile strengths similar to those of cancellous bone [253].

An interesting strategy for the controlled release of bioactive substances has been explored with poly(anhydride-esters). Bioactive substances such as antiinflammatory drugs [254], analgesics [255], and antiseptics [256] have been used as monomers or comonomers for polyanhydrides. Upon polymer degradation, the active substances were released from the polymer bulk in a controlled manner. To guide bone regeneration, salicylic acid–containing, poly(anhydride-ester)-based flexible sheets were investigated as barriers to prevent excessive BMP-2–induced bone formation [257].

Polyphosphazenes

Polyphosphazenes (Fig. 33.4M), which are polymers containing a high–molecular weight backbone of alternating phosphorus and nitrogen atoms with two organic side groups attached to each phosphorus atom, are a relatively new heterogenic class of biomaterials. Because different synthetic pathways allow for a tremendous variety of derivatives, phosphazene polymers exhibit a diverse spectrum of chemical and physical properties. As a result of this variety, these polymers are suitable for many biomedical applications ranging from templates for nerve regeneration to cardiovascular and dental uses as implantable and controlled-release devices [258–261].

The best-studied and most important route to polyphosphazenes, whose synthesis is generally more involved than that for most petrochemical biomaterials but offers unique flexibility, is macromolecular substitution. A reactive polymeric intermediate, poly(dichlorophosphazene), is typically synthesized by a thermal ring opening cationic polymerization of hexachlorocyclotriphosphazene in bulk at 250°C, which yields a polydisperse high–molecular weight product. The intermediate is reacted with low–molecular weight organic nucleophiles, resulting in stable, substituted polyphosphazenes, which in this case are also addressed as poly(organo)phosphazenes. Depending on the substituent chemistry, the polyphosphazene is more or less susceptible to hydrolysis. Biodegradable hydrophobic polyphosphazenes have been synthesized using imidazolyl, ethylamino, oligopeptides, amino acid esters, and depsipeptide groups (dimers composed of an amino acid and a glycolic or lactic ester) as hydrolysis-sensitive side groups. Hydrolytic degradation products include the free side group units phosphate and ammonia as a result of backbone degradation [258]. Hydrogel-forming, hydrophilic polyphosphazenes can by synthesized by introducing small, hydrophilic side groups such as glucosyl, glyceryl, or methylamino. Ionic side groups yield polymers that form hydrogels upon ionic complexation with multivalent ions [262]. Hydrophilic, water-soluble polyphosphazenes with amphiphilic side groups such as poly(*bis*[methoxyethoxyethoxy]phosphazene) (Fig. 33.4M, R,R′: $-OCH_2CH_2OCH_2CH_2OCH_3$) display an LCST (see the Poly(*N*-isopropylacrylamide) section) and are responsive to changes in temperature and ionic strength [263]. Both hydrophilic and hydrophobic polyphosphazenes have demonstrated potential as biocompatible materials for controlled protein delivery. Ionic polyphosphazenes have been explored as vaccine delivery systems and poly(*di*[carboxylatophenoxy]phosphazene) has demonstrated remarkable adjuvant activity in the immunogenicity of inactivated influenza virions and commercial trivalent influenza vaccine in the soluble state [258].

Porous scaffolds from biodegradable polyphosphazenes have been shown to be good substrates for osteoblast-like cell attachment and growth with regard to skeletal tissue regeneration [264]. It was also shown that hydroxyapatite deposition was supported by polyphosphazenes with side groups containing antioxidative properties [265]. Tubular polyphosphazene nerve guides were investigated in a rat sciatic nerve defect. After 45 days, a regenerated nerve fiber bundle was found to bridge the nerve stumps in all cases [259].

Biodegradable Cross-linked Polymer Networks

The chemical cross-linking of individual linear polymer chains results in networks of increased stability. This concept has been extensively explored for applications in regenerative medicine and most likely represents the concept of choice for modern biomaterial research, especially if polymer cross-linking can be conducted inside a tissue defect [266]. The cross-linking of hydrophobic polymers or monomers results in tough polymer networks that can be used for orthopedic fixation. PMMA (Fig. 33.1F), the main component in injectable bone cements, is the most prominent example. Because of their hydrophobicity, the precursors are typically injected as a moldable liquid or paste free of additional solvents. In situ cross-linking can be initiated thermally or photochemically by UV-rich light. Both ways of initiation are also applicable to hydrophilic injectable systems that form highly swollen

gels (hydrogels) as a result of precursor cross-linking. In contrast to hydrophobic networks that scarcely swell in the presence of water, injectable hydrogels are characterized by a high water content and diffusivity, which allow for the direct encapsulation of cells and sufficient transport of oxygen, nutrients, and waste. Hydrophobic networks, however, often require the addition of a leachable porogen, such as salt particles, to facilitate cell migration and tissue ingrowth. Generally, injectable polymer systems have considerable advantages over prefabricated implants or tissue engineering scaffolds, which include the ability to fill irregularly shaped defects with minimal surgical intervention [267].

A number of demanding requirements have to be fulfilled by synthetic materials for applications in regenerative medicine. In addition to physicochemical properties that fit the application site, the polymer and any adjuvant component that is required to formulate an in situ cross-linkable system have to be biocompatible. Ideally, the resulting network should also have the ability to support cell growth and proliferation early in the tissue regeneration process [53,266].

The cross-linkable synthetic polymers that will be discussed in the following sections are reactive polyesters. The main chemical functionality involved in the chemical cross-linking mechanisms is the polarized, electron-poor double bond, such as in vinylsulfones and in esters of acrylic acid, methacrylic acid, and fumaric acid. Other chemically or thermally cross-linkable macromonomer functional groups are styryl, coumarin, and phenylazide; these will not be discussed here [268].

Cross-linked Polyesters

Fumarate-based polymers: The development of fumarate-based polyesters for biomedical applications began several decades ago. Fumaric acid is a naturally occurring metabolite found in the tricarboxylate cycle (Krebs cycle); it is composed of a reactive double bond available for chemically cross-linking reactions. These characteristics make fumaric acid a candidate building block for cross-linkable polymers. The first and most comprehensively investigated fumarate-based copolymer is the biodegradable copolyester poly(propylene fumarate) (PPF) (Fig. 33.5A). PPF was first polymerized from fumaric acid and propylene oxide [269]. Mikos and coworkers optimized the synthesis of PPF and broadly investigated the tissue compatibility and applications of PPF both in vitro and in vivo [14]. Synthesis progressed to the copolymerization of fumaryl chloride and 1,2-propanediol (propylene glycol) [270]. It

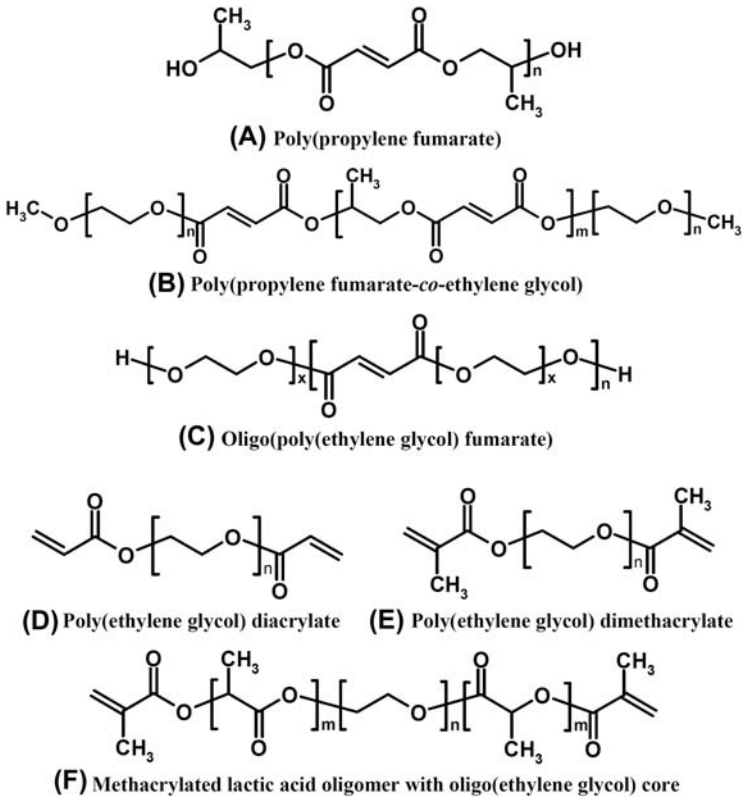

FIGURE 33.5 Chemical structures of synthetic polymers for the fabrication of cross-linked biodegradable networks.

now involves the transesterification of diethylfumarate with propylene glycol and subsequent polycondensation of the diester intermediate *bis*(2-hydroxypropyl) fumarate (PF) [271]. A variety of methods to synthesize PPF have been explored; each results in different polymer molecular weights and properties [272]. PPF has been developed as an alternative to PMMA bone cements. PPF can be injected as a viscous liquid and thermally cross-linked in vivo, eliminating the need for direct exposure of the defect site to light. Typically, PPF is cross-linked with either methyl methacrylate or *N*-vinyl pyrrolidone (NVP) monomers and benzoyl peroxide as a radical initiator [273,274]. Depending on the ratio of initiator, monomer, and PPF, the curing time can be controlled between 1 and 121 min. Compared with PMMA, which is not resorbable and is limited because its high curing temperatures (94°C) can cause necrosis of the surrounding tissue, the curing temperature of PPF has been shown not to exceed 48°C [275,276]. PPF can also be photocross-linked via the electron-poor double bonds along the backbone. Typical formulations include NVP, diethylfumarate, or PF-diacrylate (PF-DA) as comonomers together with a photoinitiator such as *bis*(2,4,6-trimethylbenzoyl) phenylphosphine oxide [277–279]. The mechanical properties of PPF, which depend on the composition, synthesis condition, and cross-linking density, are promising. However, these materials are probably not sufficient for load-bearing applications, especially when used as macroporous scaffolds [267,278,280]. One strategy to strengthen PPF scaffolds further includes the incorporation of nanoparticulate fillers. Reinforced PPF composites have been synthesized using aluminum oxide–based ceramic nanoparticles and chemically modified single-walled carbon nanotubes (SWNT). For just 0.05 wt% loading with the latter, a 74% increase was recorded for the compressive modulus and a 69% increase for the flexural modulus compared with plain PPF/PF-DA [281]. The chemical integration of alumoxane nanoparticles in cross-linked PPF/PF-DA networks resulted in a significantly increased flexural modulus [282]. Both the PPF/alumoxane nanocomposites and the PPF/SWNT nanocomposites were processed into macroporous tissue engineering scaffolds [283,284] and showed good biocompatibility in vitro [285,286] and in vivo [287,288].

Microparticulate ceramic materials such as β-tricalcium phosphate (β-TCP) were also employed as an inorganic filler to improve the mechanical properties of composite scaffolds and improve the material's osteoconductivity [289]. The composite scaffolds exhibited increased compressive strengths in the range of 2–30 MPa, and β-TCP reinforcement delayed scaffold disintegration significantly in vivo [290]. Rabbit in vivo studies also revealed the biocompatibility of photocross-linked PPF scaffolds in both soft and hard tissues [291].

PPF hydrolytically degrades at the ester bonds along its backbone. The degradation time depended on the polymer structure as well as other components such as fillers. In vitro studies identified the time needed to reach 20% original mass ranging from around 84 days (PPF/β-TCP composite) to over 200 days (PPF–$CaSO_4$ composite) [266]. Slow-degrading, porous antibiotic-releasing PPF exhibited properties suitable for craniofacial degradable space maintenance applications [292].

To broaden the application spectrum for in situ cross-linkable PPF, block copolymers with hydrophilic PEG of different compositions were synthesized. Poly(propylene fumarate-*co*-ethylene glycol) (P[PF-*co*-EG]) (Fig. 33.5B) was synthesized from PPF and PEG in a transesterification reaction catalyzed by antimony trioxide; propylene glycol was removed by condensation [293]. Behravesh et al. modified the synthesis to yield well-defined ABA-type triblock copolymers from 2 mol monomethoxy-PEG and 1 mol PPF [294]. Generally, P(PF-*co*-EG) copolymers are hydrophilic polymers with specific properties including crystallinity and mechanical characteristics that depend on the molecular weights of the individual blocks and the copolymer. As a result, platelet attachment to P(PF-*co*-EG) hydrogels was significantly reduced compared with the PPF homopolymer, which makes these copolymers candidate materials when direct biomaterial–blood contact is inevitable, such as for vascular grafts [295]. Most P(PF-*co*-EG) copolymers are amphiphiles and soluble in water; this makes them candidate materials for injectable applications. ABA-type copolymers showed thermoreversible properties comparable to other PEG-containing triblock copolymers discussed earlier. The thermogelling properties of P(PF-*co*-EG) depended on the PEG's molecular weight and salt concentration and the physical gelation temperature could be adjusted to values below body temperature [294]. In addition, the hydrophobic PPF block is highly unsaturated and available for additional chemical cross-linking, which could result in stiff cross-linked networks suitable for fabricating prefabricated cell carriers. In vitro degradation studies of macroporous, cross-linked P(PF-*co*-EG) scaffolds revealed considerable mass loss and swelling over 12 weeks. In these studies, the degradation rate depended mainly on the content of the PEG-DA cross-linker and was almost unaffected by the construct porosity. Overall, the results indicated a bulk degradation mechanism of the macroporous constructs [296]. In a subcutaneous rat model, P(PF-*co*-EG) hydrogels demonstrated good initial biocompatibility followed by the development and maturation of a fibrous capsule, which is often seen for polymeric implants [270]. Overall, the reported in vitro cytotoxicity and in vivo biocompatibility assays suggest that P(PF-*co*-EG) hydrogels have potential for use as injectable biomaterials. Fisher et al. demonstrated the suitability of thermoresponsive P(PF-*co*-EG) hydrogels for chondrocyte delivery in regenerating articular cartilage defects [297].

As previously discussed for stealthy PEG-containing biodegradables, PEG content and the hydrophilicity of cross-linked P(PF-*co*-EG) hydrogels are critical factors affecting cell adhesion [298]. Low-adhesive hydrogels enable a controlled surface or bulk modification with adhesion molecules specifically to enhance cell adhesion. P(PF-*co*-EG) hydrogels have been modified by the covalent integration of agmatine [299] and the adhesion peptide GRGDS [300]. Significantly increased numbers of smooth muscle cells and marrow stromal cells adhered compared with the unmodified networks.

An exclusively hydrophilic fumarate-based macromer is oligo(poly[ethylene glycol] fumarate) (OPF) (Fig. 33.5C). OPF macromers have been synthesized from PEG and fumaryl chloride by a simple condensation reaction in the presence of triethylamine. OPF cross-linking with or without the addition of a cross-linker such as PEG-DA can be initiated photochemically [301] or thermally [302]. In contrast to chemically cross-linked PPF and P(PF-*co*-EG), both of which form rigid polymer networks with a low water content, cross-linked OPF networks exhibit properties typical of hydrogels. Gel characteristics mainly depended on the molecular weight of PEG and the reactant ratio [301]. Cross-linked OPF hydrogels degrade hydrolytically along the ester bonds between fumaric acid and PEG, resulting in increased polymer swelling and a decreased dry weight. The weight loss of OPF hydrogels depended on their cross-linking density [303]. Studies investigating the mechanical properties revealed that cross-linked OPF hydrogels made from low−molecular weight PEG (1000 Da) swelled less, were stiffer, and elongated less before fracture compared with hydrogels composed of longer PEG chains. OPF hydrogels can also be combined in layers to form biphasic gels, with each phase having different material properties [302]. An in vitro investigation was conducted of the cytotoxicity of each component of OPF hydrogel formulation and the resulting cross-linked network employing marrow stromal cells (MSCs). After 24 h, the MSCs maintained more than 75% viability for OPF concentrations below 25% (w/v). A high−molecular weight (3400 Da) PEG-DA cross-linker demonstrated significantly higher viability compared with lower−molecular weight (575 Da) PEG-DA. Leachable products from cross-linked OPF hydrogels were found to have minimal adverse effects on MSC viability [304]. The in vivo bone and soft tissue compatibility of OPF hydrogels was demonstrated using a rabbit model [303]. Based on these promising biocompatibility data, OPF-based hydrogels were investigated as injectable drug, DNA, and cell delivery devices. Cross-linked OPF hydrogels that encapsulated gelatin microparticles were developed as a means of simultaneously delivering two chondrogenic proteins, insulin-like growth factor-1 and transforming growth factor-β1 [305], a strategy that promoted cartilage regeneration [306]. In a more complex approach involving a bilayered OPF hydrogel and the release of a chondrogenic and an osteogenic growth factor, the materials demonstrated the potential for osteochondral tissue repair [307].

Kasper et al. developed and characterized composites of OPF and cationized gelatin microspheres that released plasmid DNA in a sustained, controlled manner in vivo [308]. To control cell adhesion to the hydrophilic hydrogels, RGD adhesion peptide-modified OPF hydrogels were developed [309]. OPF hydrogels have also been shown to be useful as injectable cell delivery vehicles for bone regeneration. MSCs were directly combined with the OPF hydrogel precursors and encapsulated during thermal cross-linking. In the presence of osteogenic supplements, MSC differentiation in these hydrogels was apparent by day 21. By day 28, mineralized matrix could be seen throughout the hydrogels [310]. Hydrogel properties have been identified as affecting osteogenic differentiation within these systems [311]. Studies focused on combining cell and growth factor delivery using injectable OPF formulations [312].

Reactive cyclic acetal polymers: Current synthetic polyesters and polyanhydrides possess distinctive properties and are used extensively in clinical practice. Despite their popularity, the acidic degradation products liberated from the bulk of these polymers raise concerns regarding adverse effects and inflammation of the implantation site. In an effort to develop alternative materials, extensive research is being done to synthesize polymers that biodegrade hydrolytically without releasing pH-affecting moieties. Polymers based on acetals, cyclic acetals, and ketals degrade and form degradation products with hydroxyl, carbonyl, and/or aldehyde groups, depending on the structure of the monomers [313]. These polymers can be used for both soft and hard tissue repair. An example of a cyclic acetal-based building block is 5-ethyl-5-(hydroxymethyl)-β,β-dimethyl-1, 3-dioxane-2-ethanol (EH).

Relatively simple polymeric cyclic acetal networks can be fabricated by the radical polymerization of diacrylated EH (EHD). The resulting networks are hydrophobic; they do not swell in water and they support osteoprogenitor cell adhesion [314]. Using the traditional salt leaching technique, macroporous biodegradable scaffolds were fabricated that supported myoblast adhesion and proliferation; they were investigated for muscular tissue engineering [315]. Networks with increased hydrophilicity were designed by copolymerizing hydrophilic PEG-DA [316], generating water-swellable EHD/PEG-DA hydrogels that were formulated as an injectable system that allowed cross-linking under cytocompatible conditions and sustained encapsulated osteoprogenitor cells for up to 7 days [317]. Copolymerization with methyl-terminated PEG monoacrylate, on the other hand, resulted in nonswelling biocompatible networks with increased hydrophilicity [318].

Other groups of biomaterials are based on polyacetals and polyketals and have shown potential in drug delivery applications owing to their pH-dependent degradation [313]. The development of alternative synthetic polymers such as those described here is a critical step toward the future success of many tissue engineering and drug delivery applications.

Polymers containing acrylate, methacrylate or vinylsulfone functionalities: Precursors of cross-linked biodegradable polyester networks that bear vinylsulfone, acrylate, or methacrylate functionalities include PEG-DA (Fig. 33.5D), PEG-dimethacrylate (Fig. 33.5E), PEG vinylsulfones, diacrylated PLA-PEG-PLA block copolymers, acrylic modified PVA, methacrylate-modified dextran, and acrylated chitosan [45,99,268]. Because the last two examples are synthetic derivatives of natural macromolecules, they are not discussed further here. Besides such hydrophilic, natural macromolecules, which are considered to be candidate building blocks based on their inherent biocompatibility, PEG is the most prominent synthetic component of cross-linked polymer networks owing to its biocompatibility and inertness. As described earlier, PEG is hydrophilic and does not promote cell adhesion. To improve cell adhesion to cross-linked PEG hydrogels, adhesion peptides containing the tripeptide motif RGD were incorporated [319–321]. Research on engineered hydrogels has focused on mimicking the invasive characteristics of native ECMs by including substrates for matrix metalloproteinases (MMPs) in addition to integrin-binding sites. PEG hydrogels cross-linked in part by MMP-sensitive linkers were made degradable and invasive for cells via cell-secreted MMPs [322]. Critical-sized defects in rat crania were completely infiltrated by cells and were remodeled into bony tissue within 5 weeks when these gels were loaded with recombinant human BMP-2 and implanted in the defect site. As in natural ECMs that sequester a variety of cellular growth factors and act as a local depot for them, invading cells were presented with a mitogen that, in this case, specifically promoted bone regeneration [323]. The PEG-based hydrogels used in these studies were fabricated by a "click"-type conjugate addition reaction between vinylsulfone-functionalized branched PEG and thiol-bearing peptides under almost physiological conditions.

To enhance the initial mechanical stability and biodegradability of cross-linked PEG-based hydrogels, oligomeric biodegradable lipophilic blocks such as oligo(lactic acid) [324] (Fig. 33.5F) and oligo(caprolactone) [325], were included in the cross-linkable polymeric precursors. In a critical-sized cranial defect model, porous cross-linked scaffolds made from diacrylated poly(ethylene glycol[2]-lactic acid[10]) combined with osteoinductive growth factors showed potential as an in situ–forming synthetic bone graft material [326].

Biodegradable oligomeric macromers containing biodegradable segments were synthesized for hard tissue applications and drug delivery. To enable more flexibility in materials design and the degree of cross-linking, the central hydroxyl-bearing core molecule was changed from a linear diol such as PEG to a trivalent alcohol with different degrees of ethoxylation [327]. Oligomeric biodegradable domains were incorporated by ROP of lactides or caprolactone. Macromer reactivity for cross-linking was introduced by methacrylation of the chain termini. The degree of core ethoxylation as well as the content of lactic acid or caprolactone units per arm controlled the material properties and provided a material platform for different applications. A similar material system with higher molecular weights was developed for ocular drug delivery [328,329]. These macromers contained lactide- and trimethylene carbonate–based degradable domains and were end-functionalized for cross-polymerization with methylfumarate or methacrylate moieties. Acrylated poly(glycerol sebacate) has been used as an effective tissue adhesive [330].

Photopolymerized (meth)acrylated biodegradable hydrogels have been used in a wide range of biomedical applications. As described earlier, limited interactions with proteins are characteristic of hydrophilic surfaces. Consequently, applications such as the use of cross-linked hydrogels as a barrier applied after a tissue injury to improve wound healing or as a cell encapsulation material that immunoisolates transplanted cells capitalize on this property [99,331]. Islets of Langerhans encapsulated in PEG-DA hydrogels and transplanted to develop a bioartificial endocrine pancreas are a prominent example of the latter application. The hydrogels are permeable for nutrients, oxygen, and metabolic products and enable the entrapped islets to survive and secrete insulin that is released by diffusion. Hydrophilic tissue barriers from cross-linked polyesters such as P(EG-*co*-LA) diacrylate have been used to prevent thrombosis and restenosis after vascular injury and postoperative adhesion formation after many abdominal and pelvic surgical procedures.

Cross-linked hydrophilic polyesters are also promising depots for local drug delivery because of their compatibility with hydrophilic macromolecular drugs, such as proteins or oligonucleotides. The materials' tissue compatibility and hemocompatibility even allow for intravascular applications [332]. Drug release from cross-linked hydrogels generally can be well-controlled by adjusting swelling, the cross-link density, and polymer degradation [30,333,334].

Photopolymerized methacrylated polymer networks have also been widely explored for injectable tissue engineering [45,335]. Elisseeff and coworkers employed PEG-DA scaffolds for cartilage engineering by encapsulating

chondrocytes, MSCs, and embryonic stem cells. In these studies, the cross-linked PEG-based hydrogels served as an efficient scaffold for anchorage-independent cells and promoted tissue formation. Photogelation, which offers good spatial and temporal control of hydrogel curing, was used to control the spatial organization of different cell types within a three-dimensional system for osteochondral defect regeneration by sequentially polymerizing multiple cell and hydrogel layers. In an attempt to promote hydrogel−tissue integration, a tissue-initiated polymerization technique was developed that uses in situ generated tyrosyl radicals to initiate the photogelation of an injectable macromer solution [336].

Traditionally, photopolymerization is initiated by directly exposing materials to UV or visible light in accessible cavities or during invasive surgery. For PEG-dimethacrylate hydrogels, it was shown that light, which penetrates tissue including skin, can cause photopolymerization indirectly (transdermal photopolymerization). In vivo studies revealed that gels can be polymerized in 3 min with no harm to imbedded chondrocytes and subsequent cartilaginous tissue formation, as indicated by increasing the glycosaminoglycans (GAGs) and collagen contents [337]. In deep crevices, such as may be found in larger orthopedic defects, problems are expected to arise from limited light penetration and inconsistent photopolymerization. For those applications, thermally induced cross-linking techniques appear to be advantageous [266].

Hydrogel-forming macromonomers containing other functionalities: The development of injectable hydrophilic macromonomers that can be cross-copolymerized to hydrogels under physiological conditions using cytocompatible chemistries has become a major focus in biomaterial development. The process started with the development of protocols using photo- or heat-initiated free radical polymerization of hydrophilic, typically PEG-based macromonomers in the presence of cells [266,304]. Over the years, several alternative strategies have been explored employing specific addition reactions [6], classical bioconjugation chemistry, and “click” chemistry [338,339], as well as enzymatic conjugation [340]. Specific examples include the Michael-type addition between thiol groups of designed peptides and multiarm PEG vinylsulfone [341], or the conjugation reaction between amine groups and succinimidyl esters that was used to fabricate transparent PEG-hydrogels for ocular applications from branched PEG-succinimidyl propionates and bifunctional or multifunctional PEG-amines [342]. A more complex engineering approach was presented for the direct fabrication of biologically functionalized gels with ideal structures that can be photopatterned to generate specific microenvironments in situ, all in the presence of cells [343]. In this approach, an enzymatically degradable peptide macromer was reacted with a multiarm PEG-azide through a copper-free “click” chemistry that allows for the direct encapsulation of cells. Subsequently, biological functionalities, e.g., adhesion peptides, were introduced within the gel by a thiol-ene photocoupling chemistry in real time and with micrometer-scale resolution. Another approach to the design of biodegradable and biofunctional hydrogel hybrids focuses on integrating GAGs to synthetic polymer networks. Multiarmed PEG (star-PEG) was processed to covalent hybrid networks with GAGs, mainly heparin [344,345]. In the design of hydrogels, the importance of controlling degradation properties as well as externally triggered degradation mechanisms has become clear [346,347], in addition to optimizing the base material, in situ polymerization chemistries, and cell−material interactions.

APPLICATIONS OF SYNTHETIC POLYMERS

Synthetic polymers have a vital role in biomedical applications, including nano-, micro-, and macroscopic drug and gene delivery devices [115,348−350], orthopedic fixation devices [351], and cosmetic and prosthetic implants [98], and as artificial matrices for tissue engineering applications [101]. The interested reader may be directed to the referenced reviews that provide in-depth insight into current trends and technologies. Researchers have sought to develop and clinically explore third-generation biomaterials [1] that are designed to control protein adsorption, cell adhesion and differentiation, implant integration, and foreign body reaction, and to develop biomimetic synthetic materials [4,5,352].

CONCLUSION/SUMMARY

Synthetic biomaterials have progressed from testing “off-the-shelf” plastics not developed for biomedical purposes to a field of synergistic research by engineers, scientists, and physicians dedicated to tailoring material properties for specific applications. Current trends have shifted the focus toward biology to understand and then mimic physiological interactions and signaling.

Hydrogels, especially injectable systems, have attracted increasing attention due to the ease of their application, their structural similarity to native ECM, and their good compatibility for direct cell encapsulation because of their high water content. In tissue engineering, it is no longer believed that the biomaterial itself has to provide mechanical properties comparable to the diseased tissue; instead, the polymer has to promote defect site remodeling and tissue regeneration in vivo such that the regenerated tissue is histologically and functionally indistinguishable from the surrounding tissue. Hydrogels might be superior to hydrophobic polymers in that regard, because they can degrade more quickly, which solves the problem of the formation of nonfunctional fibrous tissue on the polymer−tissue interface. Also, hydrogel breakdown can be synchronized with cell proliferation and migration by using an enzymatically cleavable cross-linker.

Besides providing tailored degradative properties, synthetic materials for regenerative medicine should allow for minimally invasive application techniques, integrate well with the surrounding tissue, and promote cell adhesion, migration, and differentiation. The development and thorough characterization of injectable biodegradables provide the foundation for injectable tissue regeneration. Nevertheless, in situ gelation or polymerization concepts will have to be developed and optimized with regard to the cytocompatibility and stability of the resulting construct. The implementation of biomimetic design strategies will enable control and custom-designed cell−biomaterial interactions to guide tissue formation from transplanted cells. Strategies based on gene delivery or gene-activating biomaterials also have great potential in regenerative medicine, but the long-term safety of such therapies remains to be proven. Developments in stem cell biology and conjugation chemistry, such as biorthogonal "click" chemistries, have allowed for the convenient biofunctionalization of the polymeric base structures presented in this chapter with small molecules, peptides, proteins, GAGs, or DNA/RNA toward implant materials with tailored properties and bioactivity. Advances in additive manufacturing provide tools to process such engineered synthetic biomaterials into customized implants with optimized outer shapes and pore structures as well as porosity.

Overall, advances in the field of biomaterial synthesis and the design of physicochemical properties in conjunction with rapidly increasing knowledge in stem cell biology regarding adhesion, migration, differentiation, and signaling will reveal design concepts for improved injectable, biomimetic, polymer-based formulations for tissue engineering applications.

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CHAPTER

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Calcium Phosphate Bioceramics and Cements

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INTRODUCTION

With ever-increasing growth in the world's population and average life expectancy, chronic bone disorders such as osteoporosis have become more prevalent, resulting in nearly nine million bone fractures annually worldwide [1]. This development, coupled with the high prevalence of bone fractures caused by trauma, has created the need to develop novel therapies and strategies to treat fractures and augment other bone defects more efficiently and cost-effectively. Conventional approaches to overcoming these complications have involved the use of bone autografts and allografts, which provide the implant site with natural osteogenic growth and some structural support [2]. These standard treatment options have had relative success and are considered the reference standard in treatment, but several shortcomings associated with them have prompted clinicians to seek alternative strategies [3,4].

For example, the application of autografts is limited by the size of the implant site and the viability of the host tissue [4]. Furthermore, the graft will often be resorbed by the body before osteogenesis is fully complete for large bone defect sites. This phenomenon is attributed to the unpredictable bone resorption characteristics of the grafts [5,6]. Autografting also requires surgeons to harvest donor tissue, which is relatively scarce and requires a secondary procedure that increases the operating time and overall cost. In addition, this procedure runs the risk of donor-site morbidity, which can include long-lasting pain, infection, nerve damage, and hematoma once the donor graft is removed [3,4,7]. A major limiting factor of allografts relates to the immune response that is triggered by the introduction of a foreign tissue into the body. When using allografts, surgeons run the risk that the tissue may be rejected by the body and initiate an inflammatory response, resulting in complete resorption of the graft [3,7]. Transmission of disease and/or infection is also possible and is capable of causing partial or complete loss of the bone inductive factors [8].

The wide array of risk factors associated with these treatment options has prompted the need to develop alternative materials for use as bone substitutes. Furthermore, the need to improve bone filler materials for reconstruction of large orthopedic defects and the need for implants that are more suitable, both mechanically and chemically, for their in vivo environment has prompted the development of new biomaterials [4]. More specifically, calcium phosphate (CaP) bioceramics have been used as a bone substitute material to replace, augment, and promote new bone formation since the 1920s [9]. CaP bioceramics are considered to be a highly favorable bone substitute material because CaP compounds make up approximately 70% of the inorganic phase of bone, which gives these materials excellent osteoconductive and bioactive properties [10].

Calcium Phosphate Bioceramics

CaP bioceramics are usually manufactured in the form of blocks or granules that make up the bulk raw material of bone substitute materials [11]. From a chemical perspective, CaP compounds are salts that contain $H_2PO_4^-$, $H_2PO_4^{2-}$, or PO_4^{3-} ions. Of these three ions, only HPO_4^{2-} and PO_4^{3-} ions exist naturally in the inorganic mineral phase of teeth and bones [12], which gives them excellent biological properties for use as a bone substitute material for dental and orthopedic applications [13]. Table 34.1 lists CaP compounds along with their standard abbreviations,

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TABLE 34.1 Calcium Phosphate Compounds and Their Major Properties [16]

Name and Abbreviation	Chemical Formula	Ca/P Ratio	Density (g/cm^3)	Solubility at 25°C (mg/L)
Amorphous calcium phosphate (ACP)	–	1.5	3.01	25.6–32.8
Hydroxyapatite (HA)	$Ca_{10}(PO_4)_6(OH)_2$	1.67	3.16	~0.3
Calcium-deficient hydroxyapatite (CDHA)	$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$	1.5–1.67	3.16	~9.4
Dicalcium phosphate anhydrous (DCPA)	$CaHPO_4$	1	2.92	~48
Dicalcium phosphate dihydrate (DCPD)	$CaHPO_4 \cdot 2H_2O$	1	2.27	~88
Monocalcium phosphate monohydrate (MCPM)	$Ca(H_2PO_4)_2 \cdot H_2O$	0.5	2.23	~18,000
Octacalcium phosphate (OCP)	$Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$	1.33	2.61	~8.1
α-Tricalcium phosphate (α-TCP)	α-$Ca_3(PO_4)_2$	1.5	2.86	~2.5
β-Tricalcium phosphate (β-TCP)	β-$Ca_3(PO_4)_2$	1.5	3.07	~0.5
Tetracalcium phosphate (TTCP)	$Ca_4(PO_4)_2O$	2.0	3.05	~0.7

chemical formula, Ca/P molar ratio, density, and solubility data [14–16]. A more comprehensive review of the structure and chemistry of all CaP compounds can be found elsewhere [12]. Hydroxyapatite (HA) and β-tricalcium phosphate (β-TCP), or their biphasic combination, comprise most commercially available CaP bioceramics.

One of the most important properties of CaP compounds is its solubility, because this characteristic strongly influences the in vivo behavior of the bioceramics [14]. Specifically, the degradation rate, and therefore the rate of new bone formation, is directly related to the chemical solubility of the CaP compounds. Fig. 34.1 outlines the

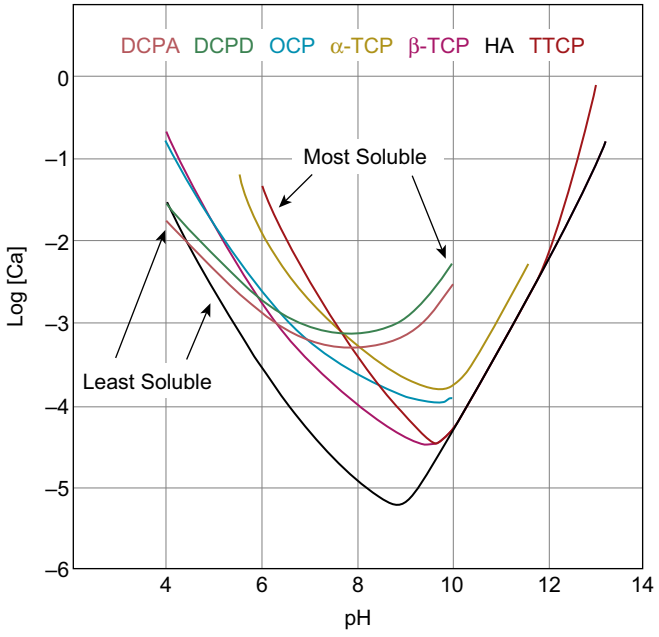

FIGURE 34.1 Two-dimensional solubility phase diagram for calcium phosphate compounds [17]. *DCPA*, dicalcium phosphate anhydrous; *DCPD*, dicalcium phosphate dehydrate; *HA*, hydroxyapatite; *OCP*, octacalcium phosphate; *TTCP*, tetracalcium phosphate; *α-TCP*, α-tricalcium phosphate; *β-TCP*, β-tricalcium phosphate.

solubility phase diagrams for all types of CaP compounds [17]. Based on these phase diagrams, it can be stated that generally, under neutral pH conditions, the degradation rate of CaP compounds follows a pattern from most soluble to least soluble [14]:

$$\text{MCPM} > \text{TTCP} > \alpha\text{-TCP} > \text{DCPD} > \text{DCPA} > \text{OCP} > \beta\text{-TCP} > \text{CDHA} > \text{HA}$$

where MCPM is monocalcium phosphate monohydrate, TTCP is tetracalcium phosphate, α-TCP is α-tricalcium phosphate, DCPD is dicalcium phosphate dehydrate, DCPA is dicalcium phosphate anhydrous, OCP is octacalcium phosphate, and CDHA is calcium-deficient HA.

By exploiting these solubility patterns, researchers are able to tailor and modify the biodegradation rate of these CaP bioceramics to better suit the rate of new bone formation. For example, biphasic CaP compounds composed of HA and β-TCP were designed by incorporating β-TCP into HA ceramics. As a consequence, these biphasic bioceramics exhibited a greater rate of resorption than pure HA ceramics [18]. More specifications regarding the biodegradability and solubility trends of CaP compounds can be reviewed elsewhere [12,19].

In the 1970s and 1980s, research into CaP bioceramics focused on their development for dental and orthopedic applications in the form of dense or porous blocks and granules [15,19,20]. Some of the most common dental applications include alveolar ridge augmentation, fillers for periodontal bony defects, tooth root replacement surgery, and coatings for metal screws for dental implants. Typical orthopedic applications included repair of bony defects as well as coatings for orthopedic metal implants usually used for hip and knee replacement surgery [19]. Although these more traditional CaP bioceramics have shown some clinical success, they still lack some important properties that severely restrict their clinical application, in particular with respect to their handling properties. CaP blocks or granules usually require invasive surgery to reach the desired implant site and achieve complete filling of the bone defect. Furthermore, because granules have poor cohesion, they run the risk of migrating out of the implant site, which can lead to unpredictable bone growth or other complications such as blood clotting [21]. From a surgeon's point of view, trying to shape granules or blocks to fit into an irregularly shaped bony defect site can prove challenging and lead to implant migration [22]. Therefore, these forms of CaP compounds have proven to be of limited value when the defect site is not easily accessible or when microinvasive percutaneous surgery would be a preferred method for implantation. Moreover, CaP in bulk form is considerably brittle in nature and exhibits poor mechanical strength and fracture toughness, which limits its uses to nonloading bearing sites [11]. These complications, coupled with the fact that most surgical trends lean toward the development of minimally invasive surgical techniques, have warranted the development of a bone substitute material that exhibits superior handling properties and can be injectable or moldable [23]. These demands have resulted in the development of calcium phosphate cements (CPCs).

Calcium Phosphate Cements

A current challenge for surgeons is to be able to place a bone substitute material directly into the defect site using the least invasive method possible. With the development of more advanced minimally invasive surgical procedures such as percutaneous surgery, there has been a demand for researchers to develop bone substitute materials that can be injectable, such as CPCs [11,24]. CPCs are a synthetic, self-setting bone substitute material that was first discovered by Brown and Chow in the early 1980s [19,25]. This "first-generation" cement consisted of an equimolar amount of TTCP and DCPA mixed with water in a liquid-to-powder ratio (LPR) of 1:4 to form a paste. This paste differed from the more traditional granule and block forms of CaPs in the sense that upon setting and hardening in vivo, the paste would sustain a rapid phase transformation resulting in the formation of HA or brushite with no hazardous acidic or basic by-products [25,26]. Details regarding this setting mechanism and the final products of the set cements are discussed further on in this chapter.

Basic Properties

The discovery of CPCs has opened up a new era for bone substitute materials in which handling properties are of upmost importance. This improvement in handling properties allowed for CPCs to be employed in minimally invasive surgery in which it also exhibited the unique ability to be molded and shaped to fill abnormal and complex defect sites where they were then able to set in situ [27]. In addition, because CPCs set in an aqueous environment at 37°C, their CDHA or brushite end product is more similar to that of natural apatites rather than CaP granules and blocks, which are sintered at high temperatures. This low-temperature formation also means that organic molecules such as polymers and living cells could be safely incorporated into the cement, which could further improve their biological response [28].

Similar to all CaP compounds, CPCs possess excellent biological properties owing to their chemical resemblance to the mineral component of bone [29]. As a consequence, several studies have shown that CPCs are highly biocompatible and osteoconductive materials with the ability to stimulate new bone growth by developing osteoconductive pathways [30]. Interestingly, it was shown that CPCs create chemical bonds to the surrounding host bone, which makes them suitable for fixating metal devices in mechanically weak bone. In addition, studies have shown that certain CPC formulations can even exhibit antimicrobial behavior as well as support osteoblast cell adhesion and gene expression in vitro [29].

CPCs also require several important properties to be employed to the best of their ability. For example, the cement requires suitable rheological properties with respect to viscosity and cohesion to ensure good bonding of the CaP particles to each other while providing adequate cell permeability. Conversely, CaP blocks and granules tend to be denser with minimal inherent porosity, which restricts their biodegradation and new bone formation. Good cohesion of CPCs is also needed to confine the CaP particles to the implant site and avoid migration throughout the body, which could lead to potential complications. CPCs also need to exhibit a suitable setting time with minimal heat output during the setting reaction to be relevant for clinical applications. Finally, the rate of resorption for the cements needs to be tailored to the rate of new bone formation so that the cement can provide adequate mechanical strength until it is replaced by new bone tissue [11,31]. CPCs are a rising class of bone substitute materials that have garnered much attention in the bone tissue engineering field. They are widely considered to be an ideal material for many bone repair and augmentation applications owing to their unique combination of biocompatibility, osteotransductivity, injectability, moldability and manipulation into bony defects, and self-setting ability in situ without producing toxic by-products. In this regard, CPCs are a more attractive material than prefabricated CaP blocks or granules for bone replacement applications [29].

CLASSES OF CALCIUM PHOSPHATE CEMENTS

In general, CPCs can be categorized into two main types based on the formed end products, i.e., apatite or brushite. The end product of the set cement is mainly determined by the solubility of the CaP precursor compounds and the pH of the setting reaction. Generally, a poorly crystalline HA or CDHA (apatite) forms when the pH value is greater than 4.2 whereas brushite, otherwise known as DCPD, forms when the pH value is less than 4.2 [32]. With respect to solubility characteristics, brushite-forming cements have a higher solubility rate than apatite-forming cements. Therefore, brushite cements tend to resorb faster than apatite cements both in vitro and in vivo [14,26].

Apatite Cements

All apatite-forming CPCs undergo a phase transformation that results in the formation of a poorly crystalline precipitated HA and/or CDHA as the final end product [32]. This end product has a strong similarity to the mineral phase in bone and teeth, which is primarily attributed to the fact that this type of cement is formed in an aqueous environment, resulting in a poorly crystalline structure. In some instances, a full phase transformation does not always occur and therefore small traces of unreacted CaP precursor compounds may remain in the matrix [29].

The most common CaP precursor compounds for apatite-forming cements include tricalcium phosphate (TCP) and/or TTCP [26]. For TCP-based cements, the particle size, degree of crystallinity, and crystal phase all strongly influence its degree of reactivity. For example, higher degrees of reactivity have been reported when the crystal phase was thermodynamically less stable. To this end, amorphous calcium phosphate (ACP) is considered to be the most reactive because it has the least stable crystal phase, followed by α-TCP and finally β-TCP [33]. Furthermore, the smaller the particle size, the more reactive the compound is, owing to the higher surface area available for reaction with the aqueous environment [32,34].

Interestingly, for apatite-forming cements, the forces holding the newly formed, highly interconnected CDHA crystals together are relatively weak, which means that detachment of the crystals in the network from each other can easily occur, especially once the passive dissolution process has been initiated. This is considered to be advantageous from biological and biodegradation points of view, because osteoclasts are better suited to ingest the smaller, fragmented apatite crystals [35]. The solubility of these cements also has an important role in regulating the biodegradation of the cements in vivo. For example, because apatite-forming CPCs and bone share a similar biological composition, they also exhibit similar solubility characteristics. Thus, like normal bone mineral, these CPCs are

relatively insoluble in aqueous solutions at a neutral pH but become more soluble as the pH becomes more acidic. This is a unique feature that allows osteoclasts to facilitate controlled dissolution during the bone remodeling process [36]. Although apatite cement degrades faster than stoichiometric HA, the rate of degradation is still relatively slow compared with brushite cements, resulting in degradation times that may take several years to decades [37].

Brushite Cements

As previously noted, all brushite-forming CPCs precipitate as DCPD as its final cementitious end product, in which the pH value of the setting reaction in the beginning is below 4.2. A wide variety of formulations have been proposed within the literature, but formulations of β-TCP + MCPM [38], and β-TCP + H_3PO_4 [29,39] tend to be most commonly used. Contrary to apatite cements, all brushite cements can form under only one type of setting reaction: the acid−base interaction, as described in more detail subsequently in this chapter. This characteristic is attributed to the chemical composition of brushite cement formulations and also the fact that DCPD can be precipitated only in a solution with a pH less than 6. Therefore, it is also understood that all formulations of this type of cement are acidic by nature during the setting and hardening phase [12,39]. For example, the pH of cements formed by a reaction between MCPM and β-TCP is initially low (∼2.5) before the pH eventually begins to rise to around 6 as the hardening phase progresses [39]. By modifying the starting formulations, the pH of the reaction, and thus several important properties of the cement, can be controlled. For example, replacing MCPM with H_3PO_4 in β-TCP−based formulations maintains a highly acidic cement paste for the first approximately 30 s of the setting reaction. Subsequently, the pH begins to rise in a similar fashion, as observed in MCPM + β-TCP formulations. Several major advantages can be obtained when MCPM is replaced by H_3PO_4 in β-TCP-based formulations, most notably: (1) more control over the reaction and chemical composition; (2) more homogeneity in the cement matrix, resulting in higher tensile strengths; and (3) prolonged setting times, which is desired specifically for brushite cements for which setting times are generally too fast [39].

The setting time of brushite cements is determined primarily by the solubility of the alkaline phase present within the CaP formulation. The more soluble the formulation, the faster the setting time is. Furthermore, a reduction in the solubility can be accomplished by increasing the alkalinity of the formulation, effectively prolonging the setting time. It is well-known that the alkalinity of HA is greater than β-TCP, which in turn is greater than α-TCP. In other words, the solubility of these three CaP compounds is: HA < β-TCP < α-TCP [14,40]. Therefore, by adding different types CaP compounds with varying pH and solubility characteristics to the formulation, the setting time can be modified. MCPM-based formulations, for instance, have been mixed with HA, β-TCP, or α-TCP. It was shown that the formulation containing HA took several minutes to set, whereas the formulation containing β-TCP took only 30−60 s to set, and finally the setting time for the α-TCP−containing formulation was further reduced to a matter of only several seconds [38]. Alternatively, incorporating additives into the cements that act by inhibiting the precipitation and growth of DCPD crystals has proven to be a successful strategy for increasing the setting time [41]. This effect has been observed for cements when setting retardants such as glycolic acid [42], sodium citrate, and citric acid were incorporated into the cement formulations [29].

Brushite cements set within a relatively short period of time from a liquid state, while these cements are highly bioresorbable and biocompatible. This latter feature is attributed to the fact that DCPD has a higher solubility and better stability under physiological conditions compared with apatite [12]. Therefore, it has been shown that brushite cements have a faster in vivo biodegradation rate than apatite cements. This rapid resorption rate also means that these cements have a rapid and significant decrease in mechanical strength after in vivo implantation [32]. Moreover, the setting times of brushite cements are generally so rapid that they require a large volume of liquid phase to keep the cement paste injectable and workable over a reasonable time. This means that high LPRs are often employed for these cements and this higher liquid content results in a more porous, inherently weaker cement as the final end product [11]. Poor mechanical strength coupled with short setting times and constricted handling properties have limited the clinical applicability of brushite cements, in particular with respect to load-bearing applications [29,43].

PHYSIOCHEMICAL PROPERTIES

Setting/Hardening Mechanism

The setting of CPCs is a continuous process that always begins with the dissolution of CaP precursor compounds into the liquid phase [17,26]. Generally, all CPCs are composed of two main phases: a solid/powder phase and a

liquid phase. Generally, the solid phase consists of one or more of CaP compounds listed in Table 34.1, whereas the liquid phase is typically either distilled water or an aqueous solution containing, e.g., phosphate-buffered saline, sodium phosphate, ammonium phosphate, citric acid, or sodium silicate [29]. Once the solid and liquid phases are combined and thoroughly mixed, dissolution of the CaP precursor compounds into the aqueous environment occurs. The rate of this dissolution highly depends on the pH of the liquid phase as well as the chemical composition of the CaP precursor compounds. Once the Ca^{2+} and PO_4^{3-} ions dissolve in solution, a supersaturation level is reached. Subsequently, these reactants begin to precipitate back onto the surface of the remaining powder constituents, usually in the form of CDHA (apatite) or DCPD (brushite) [32,44]. What is left is a highly viscous and moldable self-setting paste that can be placed directly into a bone defect site to aid in the healing process [11]. Generally speaking, once the cement is placed within the body, most of the phase transformation occurs within the first 6 h of setting; approximately 80% of the CaP precursor compounds convert to the final end products in this time frame [29].

An important factor behind the setting reaction of CPCs is the relative stability and solubility of the CaP compounds. This solubility is why a chemical transformation is induced once the CaP compound(s) are mixed with an aqueous solution. During the precipitation phase of the setting reaction, the newly formed CDHA or DCPD crystals grow epitaxially in the form of needle or rod-like microstructures that become highly interconnected and entangled. This highly entangled network of crystals provides structural rigidity to the hardened CPCs. Moreover, this highly interconnected web of crystals means that the microstructure and nanostructure of the cements possess an inherently intrinsic porosity usually between 40% and 60%, with pore sizes ranging between about 0.1 and 10 μm [11,29]. This formation of a web of needle or rod-like crystals during the setting reaction, as described previously, also leads to the adherence of crystalline grains, which results in hardening of the cement. After a sufficient time has passed, usually at least 24 h, most of the crystals have completely formed; a cement matrix is left composed of some areas of highly dense, compacted crystals whereas other areas are highly porous owing to large separations of the crystals [29].

For some apatite-forming CPCs, water is generally not employed as a reactant but rather acts as a medium. Because of this, the amount of water needed for setting to occur is minimal. Conversely, for brushite-forming CPCs, water always actively participates in the setting reaction because it is necessary for DCPD to form. Therefore, these cements are commonly referred to as hydraulic cements [30,44].

Chemical Reaction

The chemical reaction that takes place during the setting of CPCs depends on several key variables such as the pH of the reaction, the LPR, the particle size, and, most importantly, the chemical composition of the CaP precursor compounds. Two main types of setting reactions can be discerned: the acid–base interaction and hydrolysis [14,26,32].

Acid–Base Interaction

The first type of setting reaction, the acid–base interaction, employs the use of the general principles of chemistry in which, once an acidic compound and a basic compound are combined, they interact and produce a neutral compound [29]. In this instance, a relatively acidic CaP compound reacts with a relatively basic one to produce a neutral end product. A typical example of this reaction can be observed in what is considered the first CPC formulation developed by Brown and Chow in the 1980s [25]. This cement formulation consisted of acidic DCPA and basic TTCP compounds that, once combined in an aqueous liquid phase, reacted to form a relatively neutral, poorly crystalline precipitated HA, as shown in Eq. (34.1) [25,29]:

$$2Ca_4(PO_4)_2O + 2CaHPO_4 \rightarrow Ca_{10}(PO_4)_6(OH)_2 \tag{34.1}$$

It was initially thought that the reaction of DCPA with TTCP would result in the formation of stoichiometric HA (Ca/P ionic ratio = 1.67), but upon further investigation it was determined that only the first nuclei were stoichiometric HA. As the reaction continued, the nuclei would continue to grow and enlarge in the form of CDHA (Ca/P molar ratio of 1.5–1.67) crystals [25,29].

Hydrolysis Interaction

The second type of setting reaction involves the hydrolysis of CaP compounds in a liquid phase. This hydration process is mildly exothermic and is composed of five stages: (1) initiation, (2) induction, (3) the acceleration, (4) deceleration, and (5) termination [29]. Unlike the previous acid–base interaction reaction, this reaction employs the use of only one CaP precursor compound, and therefore the Ca/P molar ratio remains the same from the beginning to the end of the reaction [45]. Typical CaP precursor compounds include ACP, DCPA, CDHA, OCP, TTCP,

β-TCP, and α-TCP; α-TCP is the most commonly used in the literature. Once one of these single-phase CaP compounds is mixed with an aqueous solution, most reprecipitate into CDHA crystals during the setting of the cement. An example of the chemical reaction using α-TCP as the starting compound and water as the aqueous solution can be seen in Eq. (34.2) [29]:

$$3\alpha Ca_3(PO_4)_2 + H_2O \rightarrow Ca_9(HPO_4)\ (PO_4)_5(OH) \tag{34.2}$$

For the hydrolysis of α-TCP, the literature indicates that the reaction is controlled through the dissolution of Ca^{2+} and PO_4^{3-} ions at the surface of the α-TCP particles. Therefore, the surface area, or particle size, has a crucial role in the setting process as well as the time it takes for the cement to set fully [46,47]. Results from another study indicated that the incorporation of 2 wt% CDHA into the α-TCP powder phase to act as a nucleation sites accelerated the kinetics of the setting reaction [46]. A comparison of these two setting mechanisms, with examples from the most common CaP formulations for both apatite and brushite cements, is shown in Fig. 34.2.

Setting Times

Setting time properties are greatly important for all CPC formulations. This is especially true from the perspective of the surgeon, as the setting time dictates the amount of time the surgeon can mold and/or inject the cement as well

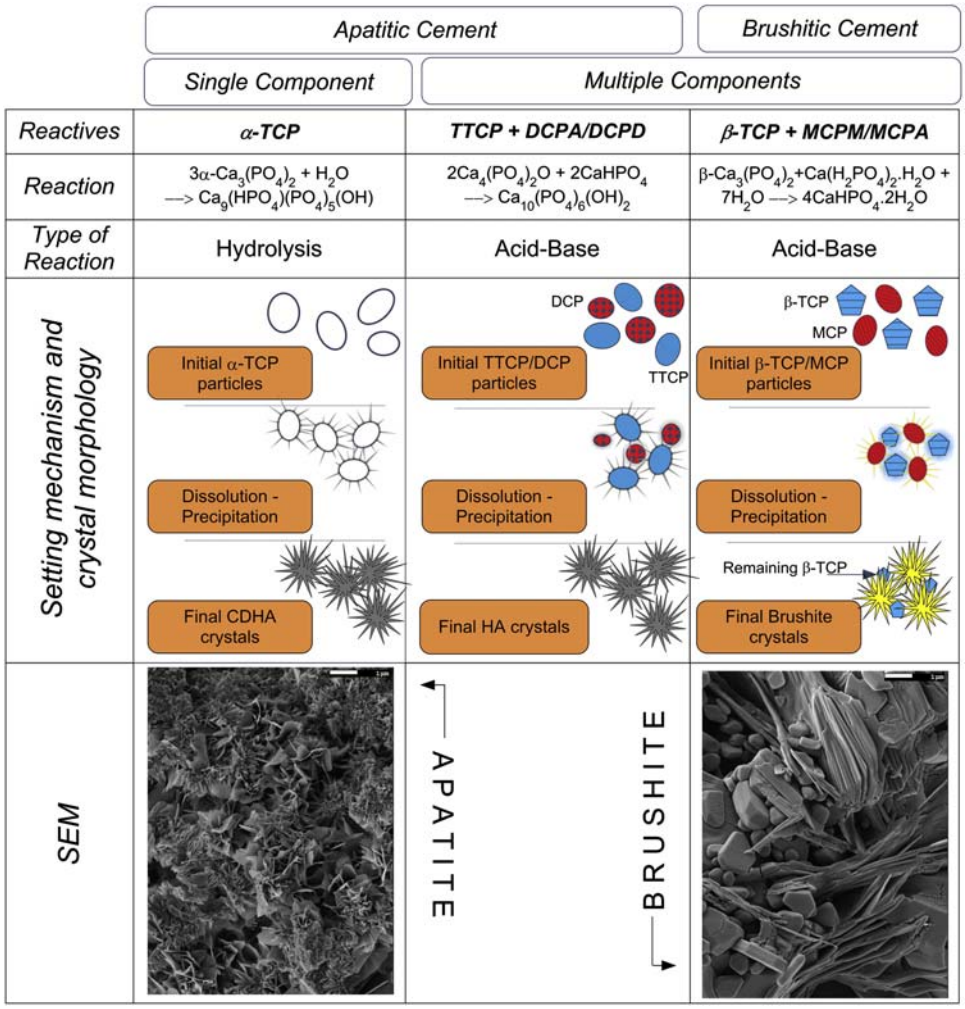

FIGURE 34.2 Classification of calcium phosphate cement setting mechanisms with examples from the most common formulations. Cements are classified based on their final phase transformation end product (apatite or brushite), the number of components in the powder phase (single or multiple), the type of setting reaction (hydrolysis interaction or acid—base interaction), and the microstructure progression during the setting reaction. *CDHA*, calcium-deficient hydroxyapatite; *DCP*, dicalcium phosphate; *DCPA/DCPD*, dicalcium phosphate anhydrous/dicalcium phosphate dehydrate; *MCP*, monocalcium phosphate; *MCPM/MCPA*, monocalcium phosphate monohydrate/monocalcium phosphate anhydrous; *SEM*, scanning electron microscopy; *TTCP*, tetracalcium phosphate; *α-TCP*, α-tricalcium phosphate; *β-TCP*, β-tricalcium phosphate. *Reprinted with permission from Ginebra M-P, Canal C, Espanol M, Pastorino D, Montufar EB. Calcium phosphate cements as drug delivery materials. Adv Drug Deliv Rev 2012;64(12):1090—1110.*

as close the wound safely in the operating theater. Setting times must be slow enough to give the surgeon time to implant and mold the cement into the defect site, and fast enough not to delay the operation. There are two main methods for how researchers can measure the setting time of the cements: the Gillmore needles method (ASTM C266-89) and the Vicat needle method (ASTM C191-92) [29]. Both involve placing a weighted needle onto the surface of the cement at different time points to visualize whether an indentation was created. Once a full indentation has not been created on the surface, the cement is considered to be set. More specifically, Gillmore needles have been used to measure the initial and final setting times of cements [48]. For instance, a lighter, thicker needle is used to determine the initial setting time whereas a heavier, thinner needle is used to determine the final setting time [49]. Both the Gilmore needle and Vicat needle methods are highly user-dependent because their results are based on the concept of "visible indentation," which can make reproducibility of data between research groups challenging. Moreover, these methods provide no information about the progression of the setting reaction that controls the setting process and time. Therefore, other methods such as impedance spectroscopy have garnered much attention as a way to monitor the setting reactions of CPCs in situ. More information regarding this technique can be found elsewhere [50].

With respect to its clinical use, the initial setting time translates to the amount of time the surgeon has to implant and work with the cement, whereas the final setting time translates to the time when the wound can be closed, as depicted in Fig. 34.3. The cement should not move or deform between the initial and final setting time because this could induce cracks. Therefore, setting time criteria have been defined as follows:

$$3\ \text{min} \leq I < 8\ \text{min}$$
$$I - CT \geq 1\ \text{min}$$
$$F \leq 15\ \text{min}$$

where CT = cohesion time, I = initial setting time, and F = final setting time. In this instance, CT is defined as the time when the cement paste no longer disintegrates in an aqueous solution [49]. The initial setting time window is relatively large and depends on the application procedure of the specific cement. For example, for dental applications, shorter initial setting times are usually preferred, whereas longer initial setting times are needed for orthopedic procedures. Regardless, the maximum final setting times for all applications generally should not exceed 15 min [49].

Strategies to Improve Setting Times

A wide array of strategies has been developed over the years to modify the setting rate of the cements so that they fall within the desired time window. Generally, brushite cements react and set much faster than apatite cements. This means that the setting reaction for brushite cements needs to be delayed whereas the setting of apatite cements needs to be accelerated to meet clinical requirements [14,40]. One of the most straightforward and common approaches toward this end is to change the particle size of the CaP precursor compounds [51]. Setting times can be significantly reduced by decreasing the mean particle size, because the size reduction increases the surface area of the particles that can then interact with the liquid phase, thus accelerating the setting reaction and hydration kinetics of the

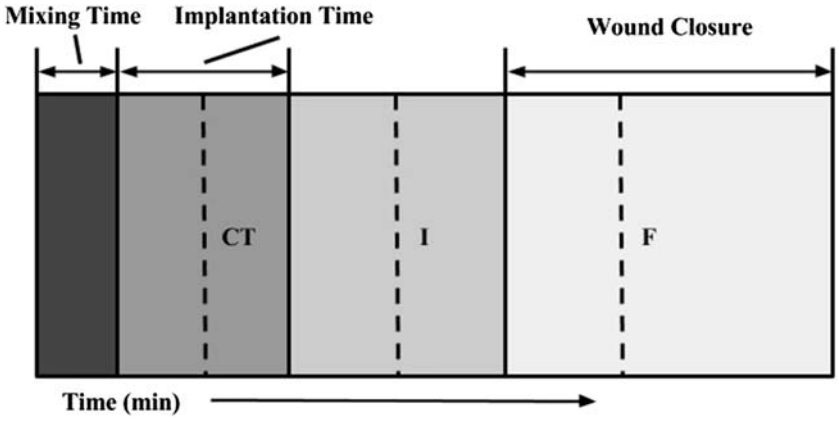

FIGURE 34.3 A diagram outlining the setting time stages of calcium phosphate cements from a clinical perspective where CT = cohesion time, I = initial setting time, and F = final setting time [29].

cement [26,34,46,47]. When this is the case, higher amounts of dissolved Ca^{2+} and PO_4^{3-} ions can be reached in a shorter time, increasing the degree of supersaturation that favors crystal nucleation and growth [52].

Another simple approach to modifying the setting time is to change the amount of liquid phase used in the cement. As is the case for most apatite cements, the amount of liquid phase used is brought to a minimum in an attempt to reduce the setting time, whereas the opposite approach is employed for brushite cements. This is one reason why apatite cements have a tendency to be more viscous and difficult to inject compared with less viscous brushite cements [26,29]. The setting time can also be reduced by incorporating soluble orthophosphates such as Na_2HPO_4 or NaH_2PO_4 into the liquid phase to reduce the pH. Creating a more acidic environment increases the solubility of the CaP compounds and accelerates the setting reaction, which shortens the overall setting time [33,53]. Furthermore, the presence of these sodium orthophosphates prevents unreacted CaP particles from becoming isolated, which could prolong the setting time [29]. Organic acids such as lactic, glycolic, tartaric, citric and malic have also been extensively used to influence the setting time of cements and have been well-described elsewhere [54].

It was also observed that for some cases, an initial thermal treatment of the CaP precursor compounds extended the setting reaction. For example, when α-TCP particles were first heated to 500°C, it increased the final setting time from the order of several minutes to upward of a few hours [55]. Finally, it is common practice to add a nucleating agent such as nanosized HA to the powder component of the cements to reduce the setting time [26,51].

Injectability

CPCs have the potential to be injectable, which sets them apart from other bone substitute materials, especially for applications involving minimally invasive surgery, in which injectability is considered a prerequisite [29]. Although there has been much debate in the literature in terms of defining injectability [15], the overall fundamental consensus is that it can be defined as the ability of the CPC paste to be extruded through a syringe, usually with a 12-gauge (i.e., 2 mm in diameter) needle 10 cm long attached to the end, although other needle dimensions have been reported [56–58]. Generally, it is measured by calculating the weight percentage of the CPC paste that can be extruded from the syringe either manually by hand or with the aid of a mechanical bench in which a force of no more than 100 N is applied [15,59].

To understand the injectability of these cements better, one must take into account the flow dynamics of the paste within the syringe. Because CPCs are composed of a powder and liquid phase, they are considered to be a biphasic material. When this biphasic material is subjected to a pressure gradient, as is the case during extrusion through a syringe, the liquid phase has a tendency to flow faster than the powder phase, which can result in local changes of the final cement composition. More specifically, because of the pressure gradient present in the syringe, the paste located closest to the plunger of the syringe is subjected to a higher pressure than the paste farther from the plunger. This higher pressure forces the liquid to flow faster than the CaP particles to the point where the paste in this region becomes depleted of liquid and all that is left is a wet powder instead of a paste [15,56,58]. The opposite effect occurs for the paste in the region farthest from the plunger, where the pressure is lower and the paste is saturated by liquid. Because these effects are dynamic, the size of the region that is depleted of liquid grows and enlarges during injection to the point where this wet powder zone eventually reaches the tip of the syringe and plugs it, rendering the remaining material noninjectable. This phenomenon, in which a phase separation occurs once pressure is applied to the paste, is commonly referred to as filter pressing [15]. As a result of the phase separation, the final composition of the extruded paste becomes compromised and not fully controllable. This is caused by the change in the LPR that takes place, which can influence the setting behavior, mechanical properties, and biological performance of the final cement product. Hence, good cohesion of the paste is of utmost importance to avoid problems associated with filter pressing [60].

Strategies to Improve Injectability

As mentioned previously, an important advantage of CPCs is their ability to be injected for use in minimally invasive surgical techniques, although this ability can become compromised when filter pressing occurs [54,56,57]. In fact, in some cases complete injectability cannot be attained even when relatively large-diameter syringe tips without a cannula are used. This implies that filter pressing can occur even under very small loading forces [11]. Thus, there is a need to develop strategies to improve the injectability of the cements. Strategies involving the addition and/or modification of certain variables have been shown to be successful for improving the injectable properties of CPCs. These variables include modifying the particle size and shape [56], the viscosity and rheology of the paste [54,57,61–64], and the LPR [15,57,65].

Particle Size and Shape

With respect to the particle size, it has been shown that the smaller the particle size, the greater the injectability [56,65]. For the particle shape, powders with a more spherical shape have been known to exhibit better injectability because the particles roll more easily and produce less shear resistance when they are extruded from the syringe. Interestingly, more spherical powders also require less liquid phase to turn into a slurry or paste, because no liquid becomes captured between the particles and therefore the liquid can be used more efficiently to wet the particles fully [11,15].

Viscosity

It is well-known that the ability of the cements to be injected is highly constrained owing to the rheological properties of the paste during extrusion. Reducing filter pressing or eliminating it from occurring by modifying the viscosity of the paste is one possible solution to improving injectability properties. One approach would be to reduce the ability of the liquid phase to pass through the powder phase. This could be achieved by increasing the viscosity of the liquid phase or by reducing the permeability of the powder phase [54,56,57,61–63]. The viscosity of the liquid phase can be modified by using additives. These additives must be nontoxic and not inhibit the phase transformation that takes place during the cement setting reaction [11].

In principle, the use of additives in the liquid phase can increase the viscosity by several orders of magnitude, resulting in the formation of a more putty-like paste. Furthermore, they can improve wettability and increase the surface charge of the CaP particles owing to the adsorption of ions on the surface [64]. Studies have shown that the addition of organic additives, such as citric acid, increased injectability by delaying the hydration time [11,66]. Furthermore, citric acid is a favorable additive for use in CPCs because it is present as citrate ions in bone and is a triprotic acid, which means that it can release or donate three hydrogen ions. This is advantageous in the sense that these citrate ions can be adsorbed onto the surface of CaP particles and give them a negative charge, and by doing so it can create a repulsion between the negatively charged particles. This repulsion enhances the mobility of the particles, increasing the injectability of the paste, because the formation of any particle agglomerations is eliminated [61]. The behavior of CPCs also depends on the type of acid additive used. For example, it was observed that the use of acetic acid, a monoprotic acid, resulted in an increase in the rate of apatite formation, which made the cement less injectable. Conversely, when citric acid was employed, the CPC became more macroporous and easier to inject, although the rate of apatite formation was reduced [59].

Another additive that was shown to improve the injectability as well as cohesion of CPCs is hydroxypropyl methyl cellulose (HPMC). HPMC, a polysaccharide gelling agent, has the ability to hydrogenate in water, forming a viscous solution in the process. This increase in viscosity improves the cohesion of the paste and makes it easier to extrude out of a syringe under minimal force with no occurrence of filter pressing [66,67]. In another study, researchers added xanthan, a type of polysaccharide, to the cement formulation to exert a lubricating effect on the interface of the CaP particles, which improved injectability [56].

Yet another technique decreases the viscosity to improve injectability. One way to achieve this is to increase the amount of liquid phase used. The idea behind this is that as more liquid phase is used, the viscosity becomes lower and the friction between the CaP particles as well as the cement paste between the syringe walls is reduced. This would make the cement easier to inject, although issues such as filter pressing are likely to occur [64,68].

Finally, the viscosity of CPCs is a constantly changing property because it is related to the setting mechanism. Traditionally, the viscosity decreases immediately after mixing the liquid and powder phases together, followed by a strong increase during the setting of the cement until hardening occurs. The viscosity needs to be at a high enough value to where extravasation of the liquid from the powder can be prevented. This can be accomplished if the viscosity falls between 100 and 1000 Pa s^{-1} and an appropriate window for injection is defined [11].

Liquid-to-Powder Ratio

Any adjustment to the LPR can have a significant influence on not only the injectability but also the setting time, porosity, cohesion, resorbability, and strength [68]. For example, high LPRs are known to increase the injectability of cements by reducing the friction between CaP particles and the syringe wall during extrusion, but this additional liquid can delay the setting time, result in poor cohesion [69], and reduce the mechanical integrity of the cement because the higher liquid content leaves behind a greater microporous structure upon dilution [11,56]. Studies have shown how the injectability of CPC formulations with LPRs between 3.85 and 4.50 g/mL have been relatively unaffected, but once this range increases to 4.50–5.00 g/mL the degree of injectability drops by nearly 100% [64]. Consequently, it seems that only major shifts in the general properties of CPCs occur once a certain LPR threshold is met.

Cohesion

Besides injectability, CPCs need to possess adequate rheological properties such as viscosity and cohesion to be employed successfully for clinical applications [15,70]. In this instance, cohesion is defined as the ability of a cement paste to keep its geometrical integrity during setting in an aqueous environment [15,26]. It can be measured by evaluating the amount of solid particles that are released from the paste under aqueous conditions, typically water or Ringer's solution before setting fully [71]. The level of cohesion is directly correlated to the degree of affinity among CaP particles within the cement, where stronger cohesive properties can result from an increase in attractive van der Waal forces and/or a decrease in repulsive electrostatic forces among the CaP particles [15]. The ideal cohesion of CPCs occurs when no disintegration of CaP particles can be observed while immersed in an aqueous environment for a period of time exceeding the final setting time of the cement [49,60]. This level of cohesion can be achieved by maintaining a high level of viscosity for the cement paste, usually through the use of gel-forming polymers or other cohesion promoters such as sodium alginate and carboxymethyl cellulose [71,72].

In the past, poor cohesive properties were associated with poor biocompatibility and negative in vivo reactions, such as inflammation triggered by the leaching and release of CaP microparticles in and around the implant site [37]. It was argued that CPCs could be considered a prime candidate for the use in vertebroplasty spinal surgery, provided that excellent cohesion of the CPC could be achieved and maintained [32]. Poor cohesive properties of CPCs used for vertebroplasty can lead to an increased risk for blood clotting. It was suggested that blood clotting is triggered by interfacial interactions between blood and CaP microparticles that fragmented off the cement and leached into the bloodstream. Coupled with the fact that vertebrae are highly perfused with blood and positioned in close proximity to the heart and lungs, this compounds the problem and exposes the patient to potentially life-threatening complications. Therefore, it is of utmost importance to maintain a high level of cohesion for CPCs to prevent the release of CaP particles and avoid in vivo complications [26].

Strategies to Improve Cohesion

The level of cohesion is directly associated with the interaction among CaP particles within the cement. Bearing that in mind, common approaches to improving cohesion are focused on reducing the mean particle size and liquid content, thus improving the van der Waal attractive forces and decreasing electrostatic repulsive forces among the CaP particles [60]. CPC pastes are considered to be non-Newtonian fluids, which means that their viscosity is a function of shear forces and time [65,70]. This poses a challenge to researchers to be able to maintain and control the viscosity, and therefore the cohesion, of the cements. However, some success has been achieved through the strategy of increasing the viscosity of the liquid phase by incorporating water-soluble polymeric hydrogels into the formulation [60,67].

Most notably, polysaccharides [32,53,67,71,72] polyacrylic acid [29], and gelatin [69] are among the most common hydrogels used owing to their biocompatibility and favorable rheological properties. Moreover, only small amounts of these polymers, on the order of just a few weight percent, need to be added to CPCs to improve their viscosity and cohesion significantly, which makes them less susceptible to washout effects [29]. In some instances, it has been shown that adding certain types of these water-soluble polymers can prolong the setting time of CPCs. To address this issue, sodium phosphate aqueous solutions have been used as the liquid phase to act as a catalyst in the setting time [73]. Other water-soluble polymers, such as sodium alginate, exhibit the unique ability to pectize (i.e., form into a jelly or gel-like substance) when placed in contact with calcium ions. This enables putty-like pastes to be fabricated, although only a few polymers displaying this unique property have been accepted for parenteral use [14,40]. Regardless, the addition of water-soluble polymers has been shown to be a promising technique to improve the viscosity and cohesion of CPCs so that the spectrum of their clinical applications can widen.

STRATEGIES TO IMPROVE THE MECHANICAL PROPERTIES

The development of CPCs has progressed significantly with respect to the optimization of their physiochemical, handling, and biological properties. Nevertheless, CPCs exhibit poor mechanical properties, because their tensile and shear strength is lower than that of teeth and bone [43]. Coupled with their inherent brittle behavior, this has limited their clinical applicability to non−load bearing or pure compression-loading sites [74,75].

The compressive strength of CPCs is the most commonly reported mechanical property in the literature, although this property offers minimal insight into the mechanical integrity and reliability of the CPC [15]. Generally, brittle materials are more likely to fail under tension or shearing rather than compression. This phenomenon is attributed

to the fact that fracturing of brittle ceramics such as CPCs initiates in preexisting flaws such as microcracks or macropores located throughout the cement matrix. It is easier to propagate a crack in tension rather than in compression [76]. Furthermore, loads within the body are generally cyclic, which indicates that fatigue properties should be reported as well [15,77]. Moreover, it is difficult to compare compressive strengths of CPCs with those of trabecular bone because bone is typically much less brittle than bioceramic cements. Therefore, it is highly recommended that the mechanical properties of CPCs be reported as more appropriate parameters such as the Weibull modulus to make a more comparative analysis between bone tissue and cement [77]. Another factor that should be taken into account regarding the mechanical properties of CPCs is that their mechanical performance in vivo may vary considerably from in vitro measurements under laboratory conditions. For example, brushite cements are highly soluble under physiological conditions compared with apatite cements, so their mechanical properties will decrease rapidly upon implantation. This sudden and spontaneous dissolution is also why brushite cements are typically combined with less soluble materials, e.g., HA or β-TCP, thus creating a biphasic CPC [15]. Based on these shortcomings, it is clear why CPCs are restricted to non−load bearing applications.

An improvement in the mechanical performance of CPCs would broaden their applicability in the operating theater to more load-bearing defect sites, e.g., spinal surgical procedures such as vertebroplasty and kyphoplasty [75,78]. To this end, the brittleness of the cements would need to be reduced along with an improvement in their fracture toughness. This toughening can be achieved by reducing the porosity of the cement matrix or combining CPCs with a polymer matrix to form a composite material [79]. Generally, these polymers are incorporated into the cement by modifying the liquid phase with polymeric additives [78] in the form of fibers [75], or by using a dual setting system in which dissolved monomers simultaneously cross-link during the cement setting reaction [80,81], as depicted in Fig. 34.4. More common formulations of various CPC−polymer composite materials have been comprehensively reviewed elsewhere [11,74].

Porosity

The mechanical strength of CPCs is directly related to their microstructural characteristics. The physical entanglement of crystals that precipitate during the setting reaction, as described earlier, results in the formation of a bulk cement matrix that determines its mechanical integrity. Several factors can affect the outcome of the cement matrix, such as the final setting products (i.e., apatite or brushite), the degree of phase transformation, the crystal size, and the porosity; with the latter being one of the most important factors. The porosity of CPCs mainly originates from the presence of an excess amount of liquid phase that has not fully reacted with the CaP particles. When this is the case, this excess liquid leaves behind open voids or pores within the entangled cement crystal matrix [78]. Generally, these pores can range from a few nanometers to upward of 8−12 μm in diameter and consume between 22% and 55% of the total cement volume [82,83]. The presence of these pores makes it much easier for microcracks to propagate throughout the cement matrix, increasing the cement porosity and decreasing the mechanical properties [29], as shown in the inverse exponential relationship [84]:

$$CS = CS_0 \exp(-KP) \tag{34.3}$$

where CS is the compressive strength of the cement at a set porosity, CS_0 is the maximum theoretical strength, K is a constant, and P is the porosity [84]. Therefore, the mechanical properties of cements can be improved by decreasing

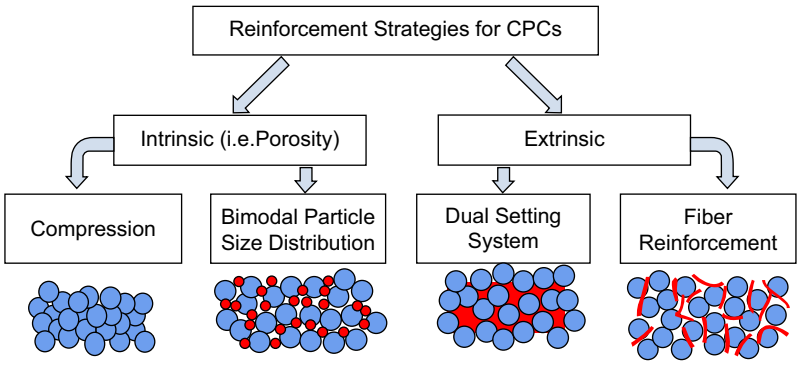

FIGURE 34.4 Mechanical reinforcement strategies for calcium phosphate cements used in load-bearing applications [78].

the matrix porosity [26]. As a rule of thumb, the tensile strength of CPCs seems to increase two-fold with every 10 vol% decrease in porosity. For example, tensile strength values of 5, 10, and 20 MPa would correlate to porosity values of 60%, 50%, and 40%, respectively [14,40]. Consequently, one method to achieve this reduction in porosity is to apply pressure to compact the CaP particles [73,84,85]. In some cases, this method has been shown to reduce the porosity from 50% to 31%, which resulted in an increase in compressive strength by almost one order of magnitude, but this method is typically unsuitable for most clinical applications [84].

Because porosity is directly attributed to the amount of liquid phase used, a more common approach to reducing it is to increase the LPR by reducing the amount of liquid phase added to the cement [14,40]. When this happens, the space between the CaP particles is reduced, enabling a more compact crystal structure to form, as illustrated in Fig. 34.5 [52]. The influence of the LPR on porosity explains why the porosity of apatite cements is usually different from that of brushite cements. The general trend is that for brushite cements the average pore size is larger than for apatite cements, but the total porosity is usually smaller than for apatite cements. This is because brushite cements have an increased water consumption during the setting reaction that also forms larger crystal sizes, which makes the average pore size greater but the total porosity smaller than apatite cements [78].

Although a reduction in the liquid content leads to a decrease in porosity, this approach is limited because all CPC formulations require a minimum amount of liquid to wet all of the CaP particles fully and create a paste [56]. Moreover, a large reduction in the liquid phase can have a negative effect on the rheological properties of the paste, such as increasing the viscosity to the point where the paste becomes noninjectable [29].

Therefore, another effective strategy for reducing the porosity of the cements is to minimize spacing between the CaP particles. This can be achieved by using a two-pronged approach: (1) create a bimodal CaP particle size distribution to fill the space among particles; and (2) create a high surface charge, or ζ-potential, on the particles. By using a bimodal particle size distribution, the smaller particles are able to occupy the space normally occupied by any

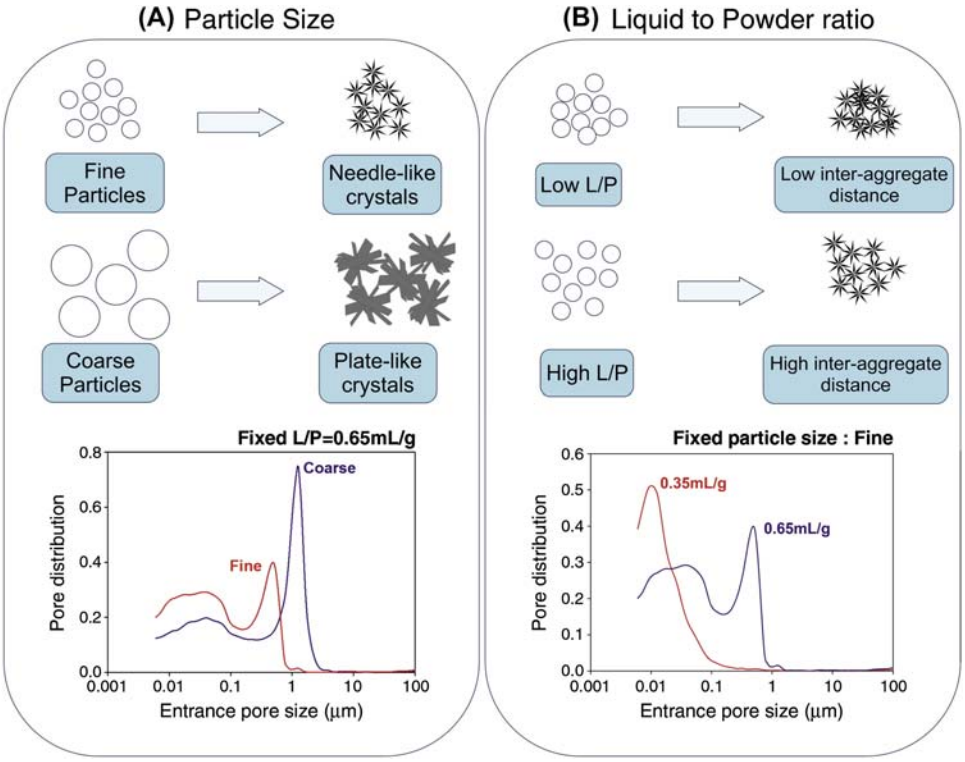

FIGURE 34.5 Schematic depiction of how the (A) particle size of the powder phase and (B) the liquid-to-powder ratio (LPR) influences the microstructure and porosity of calcium phosphate cement (CPCs). A reduction in calcium phosphate (CaP) particle size increases the particle surface area, which means a higher degree of supersaturation can be achieved that favors crystal nucleation. This leads to more frequent and smaller needle-like crystals to form instead of larger plate-like crystals that typically form when larger CaP particles are used, effectively changing the microstructure and porosity of the cement. Modifying the LPR can also fine-tune the microstructure and porosity of CPCs. Lowering the LPR reduces the spacing among CaP particles, leading to a more compact and dense crystal microstructure. The opposite effect can be seen by increasing the LPR. *Reprinted with permission from Ginebra M-P, Canal C, Espanol M, Pastorino D, Montufar EB. Calcium phosphate cements as drug delivery materials. Adv Drug Deliv Rev 2012;64(12):1090–1110.*

excess liquid, thus reducing the porosity for both apatite [86] and brushite cements [82,83]. Adding a high surface charge by increasing the ζ-potential on the CaP particles is thought to aid in dispersing and breaking up any particle agglomerates by reducing their attractive forces. This can be achieved by adding charged ions such as tartrates or citrates to the liquid phase of CPC formulations. These charged ions are then able to adsorb to the particle surface, effectively increasing the ζ-potential and removing particle agglomerations, leaving behind a more evenly dispersed particle network that exhibits smaller particle spacing [87]. Applying these two principles has been shown to increase the plastic limit (i.e., reduce the need to use more liquid) and reduce the porosity for both apatite and brushite cements. For apatite cements, a reduction in the porosity from 37% to 25% with an increase in the compressive strength from 50 to 79 MPa has been reported [86], and for brushite cements, a reduction in porosity from 30% to 23% resulted in an improvement in compressive strength from 23 to 42 MPa [82].

Finally, although a reduction in porosity leads to an improvement in the mechanical properties of CPCs, this change in the microstructure can have negative implications on the bioresorbability and osteotransductive properties of the cement. For example, the lack of an interconnected porous structure inhibits the growth of newly formed bone into the cement. This means that new bone formation would be dictated by the passive dissolution rate of the cements, resulting in a reduction in the rate of new bone formation. Therefore, it is important to understand and take into account how porosity may affect other properties of the cement, especially with respect to the biodegradability [14,40].

Dual Setting System

Another approach to increasing the mechanical strength of CPCs involves using a dual setting system. An example of this strategy involves adding polymeric components to the cement that can be cross-linked by binding Ca^{2+} ions with carboxylic acid or organic phosphate moieties within the polymer chain. The addition of the liquid phase then initiates the dual setting reaction composed of (1) the traditional dissolution-precipitation reaction of the bioceramic cement component, and (2) deprotonation of the acid groups of the polymer to induce the formation of intrachained or interchained Ca^{2+}-acid cross-links. The result of this process is a reduction in brittleness and an increase in the strength of the cement [88].

Another example of the dual setting system involves using reactive monomer units that are added to the cement formulation by first dissolving them in the liquid phase. An initiator is then added to the solid phase of the cement so that once the two phases are mixed, a simultaneous reaction is initiated that involves the traditional dissolution-precipitation setting reaction of the CaP particles and a gelation-polymerization reaction of the monomer units [78]. Within the first several minutes, a hydrogel matrix with embedded CaP particles is formed, followed by a phase transformation of the CaP particles that takes place during the setting reaction of the cement. Finally, a highly interconnected, reinforcing hydrogel matrix is formed that is located within the porous cement microstructure, as depicted in Fig. 34.6. This approach enables the possibility of adding a high amount of polymer to the cement, which translates to a potentially significant increase in its strength and toughness. Furthermore, the rheological properties of the cement paste can remain relatively stable. Both of these characteristics are attributed to the fact that the

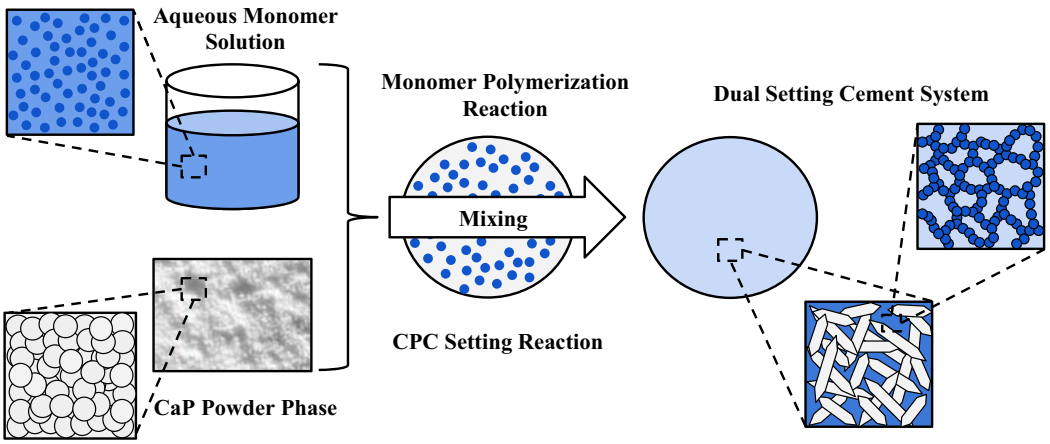

FIGURE 34.6 Schematic diagram depicting the setting mechanism of dual-setting calcium phosphate cements (CPCs) with the formation of interconnected matrices of hydrogel and precipitated calcium phosphate (CaP) crystals [78].

monomer units are small, water-miscible liquids that exhibit low viscosity such that even high concentrations of the monomers do not alter the initial viscosity of the cement [78]. Further studies regarding this concept can be found elsewhere [80,81,89].

A dual setting system can also be applied when using pure inorganic materials such as silica. Studies describing different approaches to adding silica to the cement, along with its influence on CPCs setting, handling, and mechanical properties can be found elsewhere [78,90,91].

Fiber Reinforcement

Toughening of brittle cements by using fibers has proven to be one of the most successful approaches, particularly when using long continuous fibers as a reinforcing matrix [32,75,79,92]. The mechanical performance of these fiber-reinforced CPCs depends on the complex interaction among all of the components of the composite cement [79]. The mechanical properties are also time-dependent because both the fibers and the cement matrix have the potential to degrade once they are implanted in the body to allow for tissue regeneration. These components are illustrated in Fig. 34.7, in which contributions to the mechanical behavior of the cement are associated with the strength and stiffness of the fibers and cement matrix, the toughness of the matrix, interfacial interactions between the matrix and fibers, and supplementary effects of the polymeric additives or agglomerations [79]. Excellent reviews detailing how various fiber parameters (i.e., type, volume fraction, orientation, aspect ratio, tensile modulus, and fiber–matrix interface properties) can influence the mechanical properties of CPCs can be read elsewhere [75,76,78,79].

Mechanics of Fiber-Reinforced Calcium Phosphate Cements

The concept of improving the mechanical performance of CPCs by producing fiber–cement composites was initially adopted from the field of materials engineering, where, for instance, the development of fiber-reinforced hydraulic cements and concretes for civil and industrial engineering purposes has been studied extensively. For these applications, the incorporation of fibers into the cement has been a highly effective strategy to improve fracture toughness as well as flexural and tensile strength [76].

To understand better how fibers can improve the mechanical properties of CPCs, the failure pattern of fiber-free CPCs must first be understood. Like most ceramics, CPCs are brittle materials, which means that their failure is characterized by a sudden, catastrophic fracture with minimal plastic deformation occurring before the failure point. Therefore, fracture deformation and the work needed to induce failure are relatively low. From an engineering

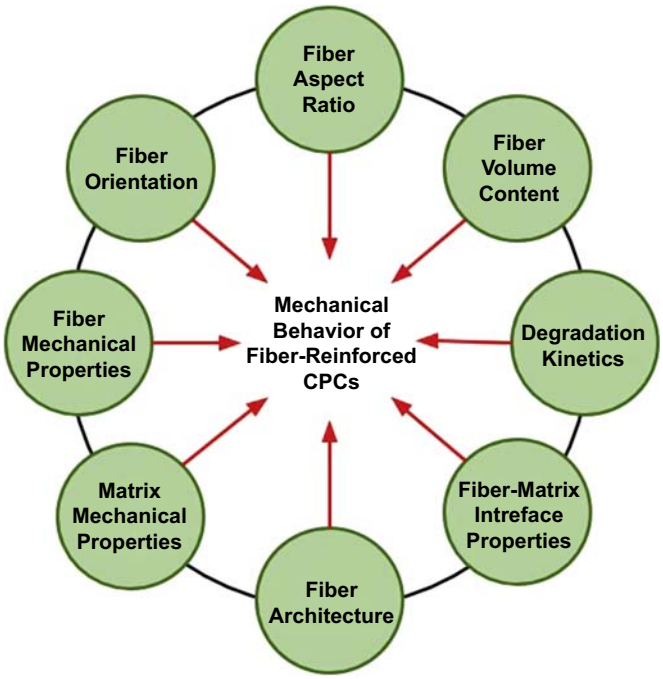

FIGURE 34.7 Illustration of the components for fiber-reinforced calcium phosphate cements (CPCs) that influence their mechanical performance [79].

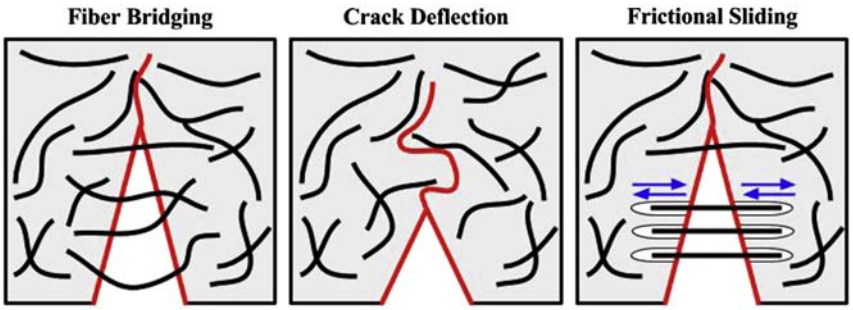

FIGURE 34.8 Schematic drawing of the three mechanisms of how fibers can mechanically reinforce cements [76].

standpoint, this brittle behavior is detrimental for mechanically loaded materials and therefore must be compensated for, in this case by incorporating fibers. The reinforcement effect of fibers in CPCs creates a tougher, more ductile cement that is more suitable for load-bearing applications because they will exhibit a higher tolerance to impact loading and sample flaws [79].

There are three main mechanisms according to how a fiber can mechanically reinforce a cement: fiber bridging, crack deflection, and frictional sliding (Fig. 34.8). In fiber bridging, fibers that bridge crack together within the matrix, thus effectively dissipating the fracture energy and delaying any further opening and propagation [79]. For crack deflection, fibers act as barriers that extend the path distance at which the crack needs to travel through the matrix. It has also been reported that these two mechanisms contribute to the high fracture toughness of human bone [93]. Frictional sliding consists of frictional sliding occurring at the fiber−matrix interface when fibers begin to pull out of the cement. This mechanism leads to a stress transfer that ultimately results in increased energy dissipation and fracture toughness of fiber-reinforced CPCs [76].

The load-bearing capacity of fibers increases with an increase in the elastic modulus as well as tensile strength. The diameter of the fibers dictates the total interface area for a given fiber volume fraction, which affects both the homogeneity and workability of the composite. Furthermore, the fiber length to diameter ratio, known as the aspect ratio, is greatly important because loading of the composite needs to be transferred from the matrix to the fibers via the interface. A reinforcing effect is observed only when the fiber length exceeds a critical value, l_c [94]. Extensive research from civil engineering revealed that the optimum fiber content to reinforce cementitious materials is typically less than 5 vol%, whereas the fiber content for fiber-reinforced CPCs is usually one order of magnitude higher. This higher fiber content is partially attributed to the fact that only a moderate load transfer has been achieved for fiber-reinforced CPCs because the interface strength has not yet been optimized for bioceramic cements [92].

Finally, because mechanical testing of CPCs is not strictly regulated, caution must be exercised when comparing mechanical values from different studies, because many variables during sample preparation and testing can strongly influence the final results [78]. For example, the strength of dried samples is typically superior to that of hydrated samples, because excess water acts as a lubricant within the entangled crystal network structure of the cement. Moreover, the macroporosity and microporosity of the cement can also be heavily influenced during sample preparation, in which any precompacting of the paste would result in a denser, less porous sample that would exhibit higher strength values compared with uncompacted samples [71,85].

CLINICAL APPLICATIONS

Oral, Maxillofacial, and Craniofacial Applications

CPC was first approved for human use by the US Food and Drug Administration in 1996. Since then, the number of CPC products and clinical indications has increased considerably, most notably in the oral, maxillofacial, and craniofacial fields [17]. CPCs are increasingly used in these fields because the CPC is stressed only moderately in these applications. Furthermore, the excellent handling properties of the CPC provide the ability to mold the material, which is highly advantageous from a surgeon's perspective. These cements have been used to repair neurosurgical burr holes, contiguous craniotomy cuts, and other cranial defects. They have also been indicated for use in sinus augmentation procedures and orbital reconstruction surgery [27,95]. Further information outlining CPCs use for oral, maxillofacial, and craniofacial applications can be found elsewhere [29].

Dental Applications

Clinical use of CPCs for dental and intraoral applications has been relatively lacking compared with their use in other fields such as craniomaxillofacial and orthopedic indications. However, research has indicated that CPCs can be highly beneficial in a number of dental and intraoral procedures. To understand better the potential of CPCs to stimulate new bone formation for dental applications, a group of researchers conducted a study in which they implanted prefabricated CPC blocks into the alveolar bone of dogs. They started by extracting all of the mandibular premolar teeth; then they waited 1 month for the alveolar bone to reduce in size to make room to implant 8-mm CPC preset blocks. Over the course of 1 month the researchers observed that the CPC block was slowly being replaced by bone. Histopathologic images further proved that the implant site exhibited features similar to those of natural bone. In addition, it was observed that the coronal half of the implanted CPC was firmly attached to the natural bone [96]. In another study, a CPC was injected into artificially created periodontal defect sites, where it was observed that the cement acted as a scaffold for new bone growth and promoted healing of the periodontal tissue [97]. CPCs have also been indicated for use in direct pulp capping, where investigators compared a self-setting CPC with that of calcium hydroxide. It was concluded that both materials were successful in producing secondary dentin after 24 weeks [98]. Finally, other studies have shown promising results for the use of CPC in restoring enamel carious cavities, for fillings in root canal procedures, for alveolar ridge augmentation, as sinus lifts, for repair of the cleft palate, and as supportive agents for dental implants [17,29]. It has been proven over the years that CPCs can exhibit superior results in these dental applications owing to their excellent osteoconductivity, unique self-hardening properties, excellent affinity to bone defect surfaces, and gradual resorption and replacement by new bone [17].

Orthopedic Applications

CPCs are predominantly used for orthopedic applications because of their distinctive handling and biological properties. The cements have been applied to a wide range of clinical procedures including: hip fractures, tibial plateau fractures, fixation of bone screws and titanium implants, vertebral body fillings and augmentation of osteoporotic-induced vertebral bodies, and distal radius fractures [36,99].

Vertebroplasty and Kyphoplasty

Two orthopedic procedures, vertebroplasty and kyphoplasty, have become increasingly popular areas in which CPCs have been employed. These procedures aim to treat osteoporosis-induced vertebral compression fractures by augmenting, stabilizing, and restoring weakened vertebra to their normal functional state and height as best as possible [100]. Vertebroplasty involves the direct injection of CPC into the fractured vertebral body, whereas an inflatable balloon tamp is used for kyphoplasty to create a cavity in the vertebral body into which CPC can then be injected to fill the cavity. Results from both procedures have shown promising results with respect to faster healing times of the vertebral body [32].

The type of CPC, and thus its properties, have a crucial role in the overall outcome and success of the procedures. For example, the CPCs must exhibit some key desirable properties such as easy injectability, excellent cohesion to prevent possible leaching of CaP particles into the bloodstream where blood clotting could be triggered, high radiopacity, mechanical properties similar to healthy vertebral bone, and a resorption rate that is comparable to normal bone remodeling times [31]. Owing to these demands, only a few CPC formulations have been allowed for clinical use in this field, and even these formulations are not necessarily considered ideal. For example, several major topics of interest still need to be investigated in more detail to optimize and tailor CPC formulations for future use. In particular, understanding the quasi-static compressive strength and fatigue performance of CPC formulations is highly interesting, especially when considering the in vivo life expectancy of the cement [32]. Overall, it has been demonstrated that the use of CPCs for vertebral bone compression fractures has substantial clinical benefits to the point where this treatment method is on the rise and is growing in popularity [11].

CONCLUSION

The rapid development of CPCs confirms that it exhibits unique characteristics that render these types of bioceramics highly suitable as a bone substitute material. CPCs are formed in a simple procedure by combining a CaP powder phase with a liquid phase to form a cementitious paste at body temperature. This paste undergoes a phase transformation at which it eventually hardens in vivo into a material that shares a composition similar to

the inorganic component found in natural bone. This self-setting capability allows for CPCs to be injectable so that they can be used in minimally invasive surgical techniques; they are also moldable so that they can adapt and fit into irregularly shaped bone defect sites. Their chemical similarity to natural bone gives them excellent osteotransductive properties. The setting times as well as cohesiveness and injectability of CPCs have improved considerably, indicating that CPCs have a promising future in the field of bone regeneration. However, the perfect bone grafting material does not yet exist, and CPCs have much room for improvement and development. In particular, CPCs are still regarded as an inadequate material for use in load-bearing sites, which severely restricts their clinical applicability. Nevertheless, many CPC products have become commercially available that are suitable for several applications, particularly in dental, maxillofacial, and craniofacial surgeries. It is anticipated that a better understanding of the fracture mechanics of CPCs will lead to an improvement in their mechanical properties, which would significantly expand their clinical relevance.

List of Acronyms and Abbreviations

ACP Amorphous calcium phosphate
CaP Calcium phosphate
CDHA Calcium-deficient hydroxyapatite
CPC Calcium phosphate cement
CT Cohesion time
DCPA Dicalcium phosphate anhydrous
DCPD Dicalcium phosphate dehydrate
F Final setting time
HA Hydroxyapatite
I Initial setting time
LPR Liquid-to-powder ratio
MCPM Monocalcium phosphate monohydrate
OCP Octacalcium phosphate
TTCP Tetracalcium phosphate
α-TCP α-Tricalcium phosphate
β-TCP β-Tricalcium phosphate

Glossary

Cohesion The ability of a cement paste to keep its geometrical integrity during setting in an aqueous environment
Injectability The ability of the CPC paste to be extruded through a syringe, usually with a 12-gauge (i.e., 2 mm in diameter) needle 10 cm long attached to the end
Osteoconduction The ability to have bone tissue grow on a material's surface
Osteotransduction The ability of a material to be resorbed and replaced by new bone tissue
Stoichiometric The quantitative relationship between reactants and products within a chemical reaction

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CHAPTER

35

Biologic Scaffolds Composed of Extracellular Matrix for Regenerative Medicine

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INTRODUCTION

The goal of regenerative medicine is to rebuild or replace damaged tissues and enable structural and functional restoration of the tissue. Many regenerative medicine strategies involve the use of scaffolds composed of synthetic polymers or biologic materials. The purposes of these scaffolds are to provide at least temporary mechanical support and to act as a temporal and spatial guide to promote cell attachment, migration, proliferation, and/or differentiation during the development of site-specific neotissues. Synthetic scaffold materials have a known chemistry that permits controlled chemical and structural modification such that they can be manufactured to almost any physical and mechanical specifications. However, synthetic materials invariably elicit a proinflammatory host response and occasionally can elicit a foreign body response. Bioscaffolds composed of extracellular matrices (ECM) as well as those composed of purified individual components of ECM (such as collagen) have also been used as surgical mesh materials. Numerous biologic scaffolds have been used in human clinical applications to treat a variety of tissue defects (Table 35.1). Biologic scaffolds are not as fine-tunable as synthetic scaffolds; however, biologic materials are typically more biocompatible and have inductive properties that can modulate cell behavior [1]. This chapter discusses the production and application of biologic scaffold materials for regenerative medicine applications with a focus on intact acellular ECM scaffolds.

EXTRACELLULAR MATRIX: FUNCTION AND COMPONENTS

ECM consists of a complex mixture of structural macromolecules and bioactive factors which not only act as scaffolding for cellular growth and motility, which gives shape to tissues, but as a bioactive, instructional environment that can regulate multiple facets of cell behavior including adhesion, migration, differentiation, proliferation, and even survival.

All multicellular organisms produce ECM although the composition can vary greatly between species. Plant ECM, for example, includes components of the cell wall, such as the polysaccharide cellulose. Chitosan, a biopolymer that has abundant commercial and biomedical applications, is derived from chitin, a nitrogenous polysaccharide that is a component of the exoskeleton of insects and crustaceans, the radulae of mollusks, the scales of fish and amphibians, and the beaks or shells of cephalopods [2]. Although these polysaccharides can be considered naturally occurring scaffold materials, they will not be discussed in this chapter. Here, the focus is on mammalian ECM, which is highly conserved among species and includes the basement membrane and the interstitial matrix between cells.

The basement membrane is a thin, fibrous, sheet-like matrix that underlies an epithelium, mesothelium, or endothelium. The interstitial matrix, on the other hand, is a gel consisting of proteins and polysaccharides that surround cells and act as a compression buffer. Resident cells produce and assemble the components of the ECM intracellularly and then secrete them into the extracellular space by exocytosis. Once secreted, these components assemble to

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TABLE 35.1 Examples of Extracellular Matrix–Based Products

Product	Company	Material	Processing	Applications
AlloDerm™	LifeCell Corporation	Human dermis	Natural, dry sheet	Soft tissue repair
AlloMax™	Becton, Dickinson and Company	Human dermis	Natural, dry sheet	Soft tissue repair
AlloPatch®	Musculoskeletal Transplant Foundation	Human dermis	Natural, dry sheet	Tendon and soft tissue repair
ArthroFlex®	Arthrex	Human dermis	Preservon proprietary preservation process	Soft tissue repair
Axis™	Coloplast	Human dermis	Natural, dry sheet	Pelvic organ prolapse and stress urinary incontinence
AxoGuard®	AxoGen	Porcine SIS	Multilaminar sheets	Nerve repair
BellaDerm	MTF Biologics	Human dermis	Natural, dry sheet	Repair integumental tissue or soft tissue
Biodesign®	Cook Biotech	Porcine SIS	Natural, dry sheet	Dura mater repair
CorMatrix®	Cook Biotech	Porcine SIS	Natural, dry sheet	Cardiac tissue repair
DermACELL®	Stryker	Human dermis	Preservon proprietary preservation process	Soft tissue repair
DermaMatrix™	DePuy Synthes	Human dermis	Natural, dry sheet	Repair integumental tissue or soft tissue
DuraGuard	Baxter	Bovine pericardium	Cross-linked, hydrated sheet	Dura mater and soft tissue repair
DuraMatrix®	Stryker	Bovine dermis	Natural, dry sheet	Dura mater repair
FlexHD®	Ethicon	Human dermis	Natural, hydrated sheet	Soft tissue repair
Glyaderm®	Euro Skin Bank	Human dermis	Preserved in 85% glycerol	Dermal repair and plastic surgery
GraftJacket™	Wright Medical Group	Human dermis	Natural, dry sheet	Tendon and ligament repair
Integra HuMend™	Integra LifeSciences	Human dermis	Natural, dry sheet	Soft tissue reconstruction
Integra® Reinforcement Matrix	Integra LifeSciences	Porcine dermis	Natural, dry sheet	Soft tissue reconstruction and tendon repair
MIRODERM®	MiroMatrix Medical Inc	Porcine liver	Sheet cut from decellularized organ	Wound repair
MIROMESH®	MiroMatrix Medical Inc	Porcine liver	Sheet cut from decellularized organ	Soft tissue repair
OASIS® Wound Matrix	Smith & Nephew	Porcine SIS	Natural, dry sheet	Wound repair
Peri-Guard®	Baxter	Bovine pericardium	Cross-linked, dry sheet	Pericardial and soft tissue repair
PerioDerm™	MTF Biologics	Human dermis	Natural, dry sheet	Dental, integumental, and soft tissue repair
Permacol™	Medtronic	Porcine dermis	Cross-linked sheet	Soft tissue repair
PriMatrix®	Integra LifeSciences	Fetal bovine dermis	Natural, dry sheet	Wound repair
SurgiMend®	Integra LifeSciences	Fetal bovine dermis	Natural, dry sheet	Soft tissue repair
Suspend®	Coloplast	Human fascia lata	Natural, dry sheet	Prolapse and stress urinary incontinence

TABLE 35.1 Examples of Extracellular Matrix–Based Products—cont'd

Product	Company	Material	Processing	Applications
TissueMend®	Stryker	Fetal bovine dermis	Natural, dry sheet	Tendon repair
Vascu-Guard®	Synovis Surgical	Bovine pericardium	Cross-linked, hydrated sheet	Vascular reconstruction
Veritas™	Baxter	Bovine pericardium	Cross-linked, hydrated sheet	Soft tissue repair
Xenform™	Boston Scientific	Fetal bovine dermis	Natural, dry sheet	Soft tissue repair
XenMatrix™	Becton, Dickinson and Company	Porcine dermis	Natural, dry sheet	Soft tissue repair
Zimmer Collagen Repair Patch®	Zimmer Biomet	Porcine dermis	Cross-linked, hydrated sheet	Tendon repair

SIS, small intestinal submucosa.

form a fibrous mesh. The composition of ECM is tissue-specific, highly dynamic, and crucially important in organ and tissue development, homeostasis, and response to injury. Each component of the ECM participates in the continuous cross-talk between cells and their environment. This general mechanism is known as dynamic reciprocity and is capable of influencing gene expression [3] (Fig. 35.1).

The ECM consists of proteins, glycosaminoglycans (GAGs), glycoproteins, and small molecules that function individually and in conjunction to support and influence cell behavior. The main components are discussed in more detail subsequently; there are excellent textbooks and reviews that describe the ECM in depth [4].

Collagen

Collagen is the most abundant protein in the human body. Fittingly, it is also the most common biologic scaffold material. The main function of collagen is to give structural support to resident cells. The domain structure of collagen is unique: a triple helix consisting of three distinct α-chains called "tropocollagen" [5]. Proper three-dimensional (3D) folding requires a glycine residue in every third position of the individual α-chains with prolines and hydroxyprolines often flanking the glycines. The helical structure is further stabilized via hydrogen bonds between the chains such that the resulting structure is highly stable.

The compliance of a tissue containing collagen depends on the form and arrangement of collagens as well as the degree of mineralization [6]. Accordingly, the 28 types of collagen have been categorized into several structural classes including fibrillar (types I, II, III, V, and XI), fibril-associated collagens with interrupted triple helices (types IX, XII, XIV, XVI, and XIX), short-chain (types VIII and X), basement membrane (type IV), multiple triple helix domains with interruptions (types XV and XVIII), membrane-associated collagens with interrupted triple helices (types XIII

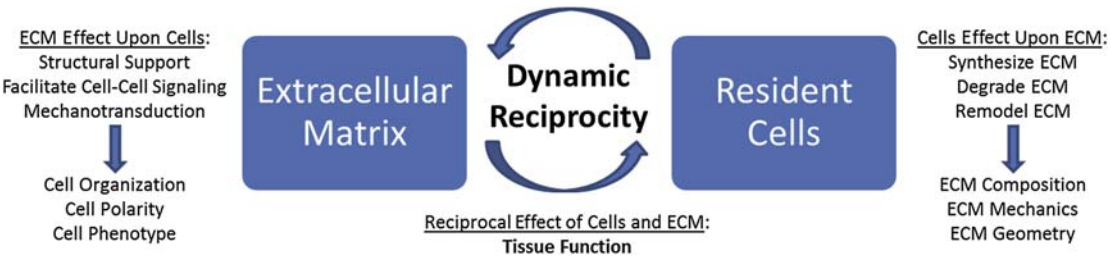

FIGURE 35.1 **ECM–cell interactions are a two-way street.** Although the ECM originates from the resident cells, the relationship between the ECM and cells is a feedback loop. Dynamic reciprocity, a mechanism whereby ECM could influence gene expression which in turn would modify and influence cell-secreted products, was proposed in 1982 by Mina Bissell [3a]. Multiple groups have since demonstrated that the ECM is able to affect cell signaling directly not only by acting as a reservoir for growth factors and other cell-secreted molecules, but also directly through mechanotransduction. Cells are constantly degrading, secreting, and remodeling their environment and thus dictate the composition, mechanics, and geometry of the ECM. In turn, the ECM is able to affect the morphology, polarity, and phenotype of cells, which ultimately affects cell and tissue function. *ECM*, extracellular matrix.

and XVII), and other (types VI and VII) [7]. Type I collagen accounts for over 90% of the collagen in the human body and is found in fibrous tissues such as skin and tendons. Fibroblasts are the most common cell type that produces collagen. In addition to its structural properties, collagen has inherent functional properties such as the stimulation or inhibition of angiogenesis and the promotion of cellular proliferation, differentiation, and attachment [8]. For these reasons, collagen is often used as a coating for cell culture vessels not only to promote cell attachment but also to influence cell phenotype.

As the main component of connective tissue, collagen is a highly conserved protein that is ubiquitous among mammalian species and accounts for approximately 25%–35% of all body proteins [9]. Inherent common amino acid sequences and epitope structures exist within collagen molecules across species [10,11]. These common antigens appear to account for the lack of an adverse immune response when xenogeneic collagen is used as an implantable scaffold material. Upon implantation, if left in its native ultrastructure, collagen implants are subjected to the fundamental biological processes of degradation and integration into adjacent host tissues. However, structural modifications such as chemical cross-linking may retard or prevent degradation and integration.

Collagen can be extracted from tissues such as tendons and ligaments, solubilized, and then reconstituted into fine strands that then can be fashioned to mimic body structures such as heart valves, blood vessels, and skin. Although collagen provides considerable mechanical strength in its natural state, the necessary mechanical and physical properties of tissue engineered products for cardiovascular and orthopedic use often require chemical manipulation of collagen-based materials. Extracted and reconstituted collagen is usually stabilized by chemical cross-linking methods and then terminally sterilized by irradiation or ethylene oxide treatment before clinical use. Chemical processing methods include glutaraldehyde treatment, carbodiimide treatment, dye-mediated photooxidation, exposure to polyepoxy compounds, and glycerol treatment. Most methods of chemical cross-linking increase the strength of collagen at the cost of decreasing the rate of in vivo degradation and negatively affecting cell attachment, proliferation, differentiation, and tissue remodeling. Exposure to chemical cross-linking agents can also change the biocompatibility of a collagen-based material, leading to a foreign body response.

Bovine and porcine type I collagen provide readily available sources of ECM scaffold material for numerous clinical applications. Isolated collagen and collagen scaffolds have been widely used in cosmetic surgery, bone grafts, artificial skin for burns, wound care, and multiple other reconstructive applications. Examples of collagen scaffolds include Contigen (Becton, Dickinson and Company, Franklin Lakes, NJ), CosmoDerm and CosmoPlast (Allergan, Inc., Santa Barbara, CA), CollaGUARD/Collieva and CollaRx (Innocoll, Inc., Ashburn, VA), Chondro-Gide (Geistlich Pharma AG, Wolhusen, Switzerland), and Menaflex (formerly Collagen Meniscal Implant [CMI]; ReGen Biologics, Inc., Franklin Lakes, NJ).

For these reasons, collagen has become a favorite substrate for many tissue engineering and regenerative medicine applications. The tissue and species source of collagen and its treatment before use are important considerations in the design of tissue engineered devices.

Fibronectin

There are two forms of fibronectin in animals: soluble plasma fibronectin and insoluble cellular fibronectin. The soluble form is produced by the hepatocytes of the liver and is a major component of blood. Deposition of plasma fibronectin at a wound site leads to the formation of a blood clot, which is critical to proper wound healing [12]. The insoluble form is a glycoprotein within the ECM. This form of fibronectin is secreted by cells, mainly fibroblasts, as a soluble protein dimer that is subsequently clustered with other fibronectins and arranged into an insoluble fibril matrix.

Fibronectin is a protein dimer consisting of two nearly identical approximately 250-kDa polypeptide chains linked by a pair of C-terminal disulfide bonds [13]. Each monomer consists of repeating units termed types I, II, and III [14]. Type I repeating units are approximately 40 amino acids long; they contain two disulfide bonds and appear 12 times in a single fibronectin. Type II units are approximately 60 amino acids long; they contain two disulfide bonds and appear twice in a fibronectin molecule. Type III units are 90 amino acids long; they contain no disulfide bonds and appear 15–17 times. The lack of disulfide bonds in type III units permits partial unfolding under applied force [15]. All three types form a β sandwich composed of two antiparallel β-sheets. Although a single gene encodes fibronectin, a number of splice variants exist, resulting in at least 20 variants in humans [16]. Splicing can occur within type III repeats, leading to "extra" units termed EIIIA (between repeats III_{11} and III_{12}) and EIIIB (between III_7 and III_8). Between III_{14} and III_{15} is a nonhomologous stretch of amino acids of variable length called the V-region, which represents another site for alternative splicing. This V-region can be partially or completely excluded, which subsequently leads to the exclusion of an integrin α4β1 binding domain. These repeating units

and splice variants are arranged into several functional domains that permit fibronectin to bind to collagen, heparin, chondroitin sulfate, heparan sulfate, fibrin, other fibronectins, and integrins α5β1, αvβ1, αvβ3, α5β6, α3β1, α8β1, αIIbβ3, and α4β1. Integrins are transmembrane receptors that facilitate cell−cell and cell−matrix interactions and can relay information to the cell about the composition and mechanics of the ECM. The sequence arginine-glycine-aspartate (known as the RGD sequence) is located within III_{10} and is the site for cell attachment via α5β1 and αVβ3 integrins. Thus, fibronectin is involved in cell adhesion, migration, differentiation, and growth.

Because of its cell-binding RGD sequence, recombinant fibronectin is often used to coat culture vessels to promote cell attachment and proliferation [17]. For these reasons, fibronectin has been conjugated to porous 3D poly(carbonate) urethane scaffolds for vascular tissue engineering and poly(D,L-lactide-*co*-glycolide) scaffolds for periodontal tissue engineering, among others [18,19]. One interesting preclinical study used modified fibronectin fragments to bind soluble growth factors and integrins simultaneously; injection of these growth factor−loaded fragments was able to improve wound healing and bone regeneration [20].

Laminin

Laminins are high−molecular weight, heterotrimeric proteins that are found in the basement membrane, specifically the basal laminae. Laminins contain an α-chain, a β-chain, and a γ-chain. There are five genetic variants of the α-chain, four of the β-chain, and three of the γ-chain. To date, 15 heterotrimers resulting from unique combinations of these chain variants have been identified in vivo, 12 of which were identified in mammals [21]. Unlike collagen, laminins do not form fibers. Instead, they self-assemble and self-bind to form independent mesh-like structures. Laminins also associate with nidogens and collagens via entactin, fibronectin, and perlecan. Through these intermolecular bonds, a network is formed that allows the basal lamina to resist tensile forces.

Heparan sulfates, β1 and β4 integrins, dystroglycan, and other surface receptors are able to bind directly to laminin moieties. The binding of laminin to these receptors and receptor-like proteins facilitates cell adhesion, migration, and differentiation [21].

With regard to the use of laminin as a biologic scaffold, laminins are a critical component of matrix materials generated from the basement membrane of tissues via decellularization, a topic that will be discussed later in the chapter. On their own, laminins have been used as a substrate for culturing cells in vitro. Two groups demonstrated that mouse embryonic stem cells can be grown on recombinant laminin-511 for long-term culture [22,23]. Human embryonic stem cells and induced pluripotent stem cells have also been grown with recombinant laminin 511 without the need for a feeder layer [24].

Elastin

As its name implies, elastin lends elasticity to tissues so that it can return to its original shape after stretching or contracting. Elastin is particularly prevalent in the ECM of the lungs, skin, ligaments, and blood vessels, where physiological forces result in intermittent stretching. Similar to fibronectin, elastin is synthesized from only one gene, but it can undergo extensive alternative splicing, resulting in tissue-specific elastin variants. Fibroblasts and smooth muscle cells secrete a soluble, nonglycosylated, hydrophobic form of elastin known as tropoelastin. These elastin precursors undergo posttranslational modification and are packaged inside chaperone molecules and secreted. In the extracellular space, tropoelastins covalently bind by patterning alternating hydrophobic and hydrophilic sequences to form a highly insoluble, cross-linked array, a process known as coacervation [25]. The tropoelastin coacervates are associated with microfibrils, particularly fibrillin-1, by the molecules fibulin-5 and fibulin-4 [26]. Lysyl oxidases cross-link the tropoelastins; after the formation of a multitude of cross-links between the tropoelastin monomers, the resulting elastin polymer becomes an insoluble fiber [27].

Electrospun recombinant human tropoelastin has been evaluated for use in wound healing [28]. Commercially, it is marketed as a product called Dermalastyl or Elastatropin for use in skin care to prevent wrinkles.

Glycosaminoglycans/Proteoglycans

GAG are long unbranched polysaccharides consisting of a repeating disaccharide unit. GAG are classified into four groups based on their core disaccharide: heparin/heparin sulfate, chondroitin sulfate/dermatan sulfate, keratan sulfate, and hyaluronic acid (HA). All GAG are synthesized in the Golgi apparatus except HA, which is synthesized at the cell membrane. Unlike protein or nucleic acid synthesis, GAG production is not template driven. GAG

are highly heterogeneous because they are subjected to dynamic modification by processing enzymes [29]. These modifications include sulfation and epimerization, which make GAG highly polar and allow them to attract water molecules and bind cations such as calcium, potassium, and sodium. Thus, GAG are able to act as a physiological lubricant or shock absorber.

One or more GAG covalently bonded to a protein are considered a proteoglycan. Proteoglycans are heavily glycosylated proteins. The protein core of the proteoglycan is synthesized in the rough endoplasmic reticulum and then posttranslationally modified with glycosylations in the Golgi apparatus. A tetrasaccharide bridge connects a GAG to a serine residue of a protein core.

Chondroitin sulfate is the most prevalent GAG. It is usually found as part of a proteoglycan, and its function largely depends on the protein to which it is attached. As part of a large aggregating proteoglycan, such as aggrecan, neurocan, versican, or brevican, chondroitin sulfate is critical to maintaining the structural integrity of a tissue. Aggrecan is a major component of cartilage in which chondroitin sulfate lends its highly charged sulfate groups to attract water and provide electrostatic repulsion to generate resistance to compression. Chondroitin can be extracted from the cartilage of cow and pig tissues for use as a biologic. Thus far, it has been investigated as a dietary supplement, drug, or injection in clinical trials to alleviate osteoarthritis; however, the symptomatic benefit is reported to be minimal [30].

Dermatan sulfate is distinguished from chondroitin sulfate by the presence of iduronic acid instead of glucuronic acid. Like chondroitin sulfate, dermatan sulfate is found in proteoglycans such as decorin, biglycan, and versican. Dermatan sulfate is mostly found in skin, but it is also present in tendons, blood vessels, heart valves, intestinal mucosa, and lungs, where it may have a role in coagulation, wound repair, response to infection, differentiation, morphogenesis, cell migration, carcinogenesis, and fibrosis [31]. Although the clinical application of dermatan sulfate is limited, companies such as Bioibérica (Barcelona, Spain) manufacture and sell it for potential use in enhancing wound repair or preventing coagulation.

Keratan sulfate is found in cornea, bone, nervous tissue, and cartilage, where it is part of proteoglycans such as lumican, mimecan, aggrecan, osteoadherin, keratocan, and fibromodulin. Like other GAG, it is highly hydrated and is able to absorb shock experienced by joints. Keratan sulfate has been demonstrated to be absent or aberrant in macular corneal dystrophy [32]; however, keratan sulfate has no widespread clinical uses.

Heparan sulfate is found in all animal tissues in various proteoglycan forms. Transmembrane heparan sulfate proteoglycans such as syndecans and glypicans are critical to cell signaling processes. Other proteoglycans are integral to the basement membrane, such as perlecan and agrin. Heparan sulfate can also be bound to a variety of extracellular proteins including collagen XVIII, growth factors, chemokines, cytokines, enzymes, and coagulation factors. Thus, heparan sulfate has a critical role in regulating developmental and biologic processes. Largely based on its ability to bind growth factors and cytokines, heparan sulfate and its mimics have been reported to promote wound repair and tissue regeneration [33,34].

Unlike other GAG, HA (also known as hyaluronan) is a polysaccharide that is not sulfated and is not found as a proteoglycan. HA is synthesized by integral membrane hyaluronan synthases that directly extrude it into the extracellular space [35]. HA is able to absorb significant amounts of water to act as a swelling force that permits tissues to resist compression. Thus, it is most commonly found in load-bearing joints and in the ECM of tissues such as skin, cartilage, connective tissue, epithelial tissue, and neural tissue [36,37]. HA is also found in nonanimal sources such as roots and tubers (potatoes and sweet potatoes) [38]. In addition to mechanical support, HA influences cell proliferation and migration. Moreover, HA facilitates these cellular functions during the late stages of wound healing and acts as a promoter of inflammation in the early stages [39]. HA has been extensively investigated as a natural scaffold material for tissue reconstruction. It has been used clinically in an injectable form for treatment of osteoarthritis of the knee, for cosmetic surgery as a dermal filler, and for ophthalmic surgery, among other applications. Examples of HA-based materials include Belotero (Merz Aesthetics, Raleigh, NC), JUVÉDERM (Allergan, Inc.), Hyalomatrix, Hyalofill, Hyalogran, Hyalosafe, and Elevess (Anika Therapeutics, Inc., Bedford, MA), and Restylane and Restylane Perlane (Q-Med Corporation, Fort Lauderdale, FL).

Matrix-Bound Nanovesicles

Matrix-bound vesicles (MBV) are a component of the ECM. They were first identified by Huleihel et al. as nanovesicles embedded within ECM scaffolds derived from porcine urinary bladder matrix (UBM), small intestinal submucosa (SIS), and dermis [40]. Like exosomes, MBV are nanosized, lipid membrane–bound vesicles with a size distribution from 10 to 200 nm. MBV were isolated from acellular porcine tissues by enzymatic digestion followed

by ultracentrifugation at increasing *g* values. MBV contain distinct profiles of microRNAs and proteins with some mutual cargo among the three ECM scaffolds evaluated. In vitro, MBV can recapitulate some of the regenerative effects observed when cells are treated with ECM, which highlights the potential of MBV as a key mechanism mediating the regenerative abilities of ECM scaffolds. For example, Huleihel et al. observed neurite outgrowth in neuroblastoma cells treated with MBV and activation of macrophages toward a phenotype associated with a more regenerative, anti-inflammatory, constructive remodeling response.

MBV may be useful for direct injection to a damaged site to encourage local anti-inflammatory and pro-remodeling pathways, or as an additive to enhance implanted scaffold performance. Identification of the variability and potential modification of MBV cargo will be critical to developing clinically relevant strategies with MBV.

INTACT AND SOLUBILIZED EXTRACELLULAR MATRIX AS A SCAFFOLD MATERIAL

Tissue Procurement

Intact ECM can be isolated from virtually any tissue. Decellularization protocols have been established for heart valves, blood vessels, skin, nerves, skeletal muscle, tendons, ligaments, ovaries, testes, small intestine, urinary bladder, liver, and other tissues. ECM is routinely harvested for research purposes and for clinical application from different species including humans, pigs, cows, and horses [1]. ECM may alternatively be collected from cells grown in vitro [41]. ECM scaffold materials harvested from different species, tissues, or cells have unique structural, functional, and biochemical characteristics. For instance, ECM scaffolds derived from porcine SIS (SIS-ECM) consist of about 90% collagen, most of which is collagen type I, and minor amounts of other collagen types III, IV, V, and VI [42]. On the other hand, although they contain the same collagen types as SIS−ECM, ECM scaffolds composed of porcine UBM (UBM-ECM) contain greater amounts of Col III as well as Col VII, which are important components of the epithelial basement membrane [43]. ECM from various sources also differ in the amount and distribution of GAGs, including heparin, heparin sulfate, chondroitin sulfates, and HA [36,37]; adhesion molecules such as fibronectin and laminin [43,44]; the proteoglycan decorin and the glycoproteins biglycan and entactin [1]; as well as various growth factors including transforming growth factor-β [45], basic fibroblast growth factor (b-FGF) [46], and vascular endothelial growth factor (VEGF) [47]. In addition, ECM scaffolds are distinctive in their protein composition from location to location within various tissues: for example, endocrine versus exocrine loci within the pancreas, or, the valvular versus mural loci within the heart. It is assumed that preservation of the intact ECM composition as well as its intrinsic ultrastructure and 3D architecture, especially its collagen fiber architecture, are fundamentally important in processes such as cell recruitment, migration, proliferation, and differentiation during neotissue formation in vivo [43,48].

Decellularization

Conceptually, intact ECM consists of all of the structural and functional components secreted by the resident cells in their native 3D microarchitecture. In reality, the content and microarchitecture of intact ECM are invariably altered during the decellularization process. ECM will necessarily lose some of its functional components as they are washed out by detergents or other liquid washes. The reagents used in these washes are occasionally retained to a small degree in the final ECM product. Moreover, various decellularization processes and reagents are known to alter the orientation, porosity, or fiber size of the main collagen network differentially, potentially affecting biocompatibility and bioactivity. Decellularization processes are designed to balance the removal of cellular material with the preservation of the composition, mechanical integrity, and biological activity of the remaining ECM. Decellularization processes attempt to achieve the metrics of decellularization established in 2011 by Crapo et al.: less than 50 ng of double-stranded DNA per milligram of dry weight ECM, no visible nuclei in hematoxylin eosin−stained histological sections, and any residual DNA being fewer than 200 base pairs in length [49]. The process from native tissue to ECM varies dramatically between tissue types, considering factors such as tissue density, vasculature, and lipid content. Decellularization processes necessarily include multiple steps, i.e., liberation of desired tissues from surrounding tissues, decellularization (several methods are described subsequently), disinfection, lyophilization, and terminal sterilization. Most commercially available intact ECM scaffolds are processed into a sheet form before decellularization by methods that include trimming and spreading of the original tissue to facilitate the removal of cellular components and debris. In addition, the decellularization of whole organs has been achieved through

perfusing detergents or other solutions through the tissue's native vascular network [50]. Common approaches to decellularization include a combination of physical and chemical treatments, e.g., sonication, agitation, freeze-thawing, and washes with various proteolytic detergents and solvents (reviewed in Gilbert et al. [51]). The decellularization process effectively removes xenogeneic and allogeneic cellular antigens that may be recognized as foreign by the host and results in an adverse inflammatory response or overt immune-mediated rejection [52,53]. The main ECM components are highly conserved between species and are well-tolerated by xenogeneic recipients [10,11]. Over 10 million patients have received ECM-based materials in clinical settings, most of which are of xenogeneic origin, and no immune rejection complications have been reported. Residual amounts of DNA and certain immunogenic species-specific antigens, such as galactosyl-α-1,3-galactose (α-Gal epitope), have been shown to be present in ECM scaffolds but fail to activate complement or bind immunoglobulin M antibody, possibly owing to the small amount and widely scattered distribution of the antigen [54–57].

Postprocessing

After decellularization, it is often desirable for the ECM to be processed further. Such processing may include lyophilization (freeze-drying) or vacuum pressing before terminal sterilization to avoid leaching of soluble factors (for example, VEGF and b-FGF), and extend the product's shelf life. The production of multilaminate forms of the ECM also improves the device's handling and allows various geometric constructs to be built, including tubes [58], cones [59], and multilaminate sheets [60,61]. Lyophilized scaffolds can be processed further to yield powdered ECM, solubilized ECM, or ECM in hydrogel form [62] for use as minimally invasive injectable scaffolds [63,64] or in combination with sheets of ECM to produce sheet-powder hybrid scaffolds. Whereas each of the processing steps will change the overall composition and structure of the prepared ECM compared with those found in vivo, intact ECM preparations retain a multitude of structurally and functionally active proteins [61,65].

ECM scaffolds that are not chemically cross-linked are rapidly degraded in vivo. Typically, 50% of a noncrosslinked SIS-ECM scaffold is degraded within one month postimplantation and the scaffold is usually completely degraded within a three month time frame, as demonstrated in the repair of a urinary bladder defect or the Achilles tendon [66,67]. ECM degradation leads to an initial decrease in overall strength during the early phase of in vivo remodeling, followed by an increase in strength caused by the deposition of site-specific ECM and the formation of functional site-appropriate neotissue by infiltrating cells in response to their experienced mechanical stresses [58,66,68,69]. Soluble factors within ECM scaffold materials (that is, growth factors, and the release of biologically active cryptic peptides resulting from degradation of the ECM material [8,70]) are thought to be directly involved in the processes of neotissue formation including angiogenesis, mononuclear cell infiltration, cell proliferation, cell migration, and cell differentiation [45,71,72]. The release of soluble factors along with the rapid degradation of the ECM appear to be essential processes for constructive remodeling to occur. This fact is highlighted by an altered remodeling profile in clinical applications using scaffolds that have been chemically cross-linked using glutaraldehyde, carbodiimide, or hexamethylene-diisocyanate, or nonchemical methods. Whereas chemical cross-linking can increase the mechanical strength and reduce the rate of scaffold degradation of an ECM scaffold, this modification results in the formation of a chronic, proinflammatory, foreign body type of tissue response and a reduced level of constructive remodeling [71,73]. Similar to the host response to synthetic polymer scaffolds, an adverse host response is heralded by a predominance of M1-like activated macrophages, a high level of proinflammatory cytokines, and the formation of a type 1 T helper (Th-1) (cell-mediated rejection) response [74]. In contrast, intact, nonchemically modified ECM scaffold materials show a host immune response characteristic of accommodation and integration, as demonstrated by the increased presence of M2-like activated macrophages resulting in low levels of proinflammatory cytokines and the establishment of a Th-2 type of response [75–77]. The initial host immune response to ECM scaffolds appears to be critically important in determining subsequent processes including scaffold degradation, release of matricryptic peptides, host cell recruitment, and angiogenesis, among others [8,72].

Hydrogels

A form of ECM that is receiving increased scientific and clinical attention is the ECM hydrogel. Unlike powder or sheet forms, ECM hydrogels are injectable, which makes them minimally invasive; and by virtue of their liquid state at room temperature, they can fill irregularly shaped volumes before they self-assemble into a gel at body temperature. The mechanical stiffness as well as the polymerization time of the resulting gel is fine-tunable by altering the concentration of solubilized ECM in the solution, which makes it a physically versatile biomaterial. Commonly used terminal sterilization methods including γ- or electron beam irradiation and ethylene oxide exposure have been shown to inhibit ECM hydrogel formation. However, supercritical CO_2 sterilization may permit sterile ECM

hydrogel formation [78]. ECM hydrogels have the benefit of retaining the inherent bioactivity of the native matrix despite the loss of its native 3D microarchitecture during the solubilization process [79]. Hydrogels are typically created using powdered ECM as a starting point and then enzymatically digesting the structural components until complete solubilization is achieved. Pepsin is popularly used to solubilize ECM, and it has been observed that proteolysis with other enzymes (papain, for example) can impede the polymerization behaviors of the resulting solution. Similar to solid ECM scaffolds, hydrogels will degrade under physiological conditions, typically within one month of implantation, depending on the source ECM and concentration, and it is supposed that this degradation is essential in achieving their favorable host response [79,80]. Many studies suggest that hydrogels from various source ECMs have vast potential to regenerate bone, adipose tissue, skeletal muscle, and even central nervous system tissue [81–83]. Ventrix, Inc. developed a hydrogel for cardiac repair and regeneration called VentriGel. To date, Ventrix is in Phase I clinical development for VentriGel.

Host Response

A unique feature of intact ECM compared with other types of bioscaffolds is the favorable host response that it elicits. As stated previously, over 10 million patients have received xenogeneic ECM-based materials in clinical settings and no immune rejection complications have been reported. The lack of an adverse immune response to the use of xenogeneic collagen in implantable scaffold materials has been attributed to the common nature of amino acid sequences and surface epitopes among species [10,11] although work has shown the immunomodulation effects of ECM degradation products [70]. Allogeneic and xenogeneic collagen is subjected to the fundamental biological processes of degradation and integration into adjacent host tissues when left in its native ultrastructure. However, structural modifications designed to alter the rate of degradation and remodeling (e.g., cross-linking) may impair the desired healing and regenerative response.

Beyond simply being tolerated by the host, intact ECM has been implicated in directing functional remodeling in many tissue types including skin [84], tendon and ligament [60], urinary bladder [85], skeletal muscle [86], heart [87], and esophagus [58]. Xenogeneic ECMs used clinically are typically of porcine origin; they are usually derived from the urinary bladder, small intestine, or dermis. In vitro, the biologic effect of different source ECMs can be diverse, which can be partly explained by the various methods used to decellularize different tissues [88]. In clinical settings, certain source ECMs are approved for specific applications and not others, although in a clinical trial for volumetric muscle loss including 13 patients, no distinguishable differences were found between patients receiving ECM from urinary bladder, small intestine, or dermis [89].

The exact mechanisms of action for ECM's favorable host response are unknown, although several observations provide a partial explanation. ECM has been shown to attract and/or facilitate differentiation of endogenous stem or progenitor cells [72], enhance mitogenesis in certain cell populations [90], and shift the innate immune response away from inflammation and toward remodeling [73]. In the clinical trial mentioned earlier, it was noted that the ECM scaffolds attracted muscle and nerve progenitor cells to the wound area, thus assembling the key players for muscle regeneration. In another study, an ECM hydrogel was applied to the volumetric lesion of a stroke infarct and within 24 h the host cell infiltrate was characterized. Approximately 60% of the cells were found to be of brain-derived phenotypes, including neural progenitor cells, which upon histological staining appeared to be differentiating into neuronal networks [81]. Another 30% of infiltrating cells were peripheral macrophages; importantly, the ECM hydrogel directed their phenotype away from the M1, classical proinflammatory activation and toward the M2, alternatively activated anti-inflammatory phenotype, putatively aiding in remodeling.

It is known that ECM and their degradation products have various effects on other immune cells as well. For example, ECM components such as entactin, nidogen, and fragments of laminin, elastin, and type IV collagen have been shown to attract neutrophils, a microbicidal cell type [91,92]. It was also found that tumor necrosis factor-α linked to fibronectin affected the signal transduction and cell adhesive properties of $CD4^+$ T cells, possibly helping to direct these immune cells toward inflammatory sites [93]. Another study showed that a 3D collagen matrix was essential for naive T cells to interface meaningfully with antigen-presenting dendritic cells, leading to signal induction and T-cell activation [94]. These studies illustrate how ECM bioscaffolds promote regenerative conditions and tissue development by priming the local immune response.

Whole-Organ Scaffolds

In 2008, perfusion of detergents through an intact donor heart was used to generate a 3D heart ECM scaffold [50]. This detergent-perfusion decellularization technique has since been applied to liver, lung, pancreas, kidney, and even a whole limb [95–99]. Whole-organ engineering using porcine-derived organ scaffolds seeded with

patient-derived stem cells provides an alternative to traditional transplantation that circumvents the need for immunosuppression and the shortage of organs available for traditional transplantation. Recellularization of organ scaffolds is the current focus of the field. To facilitate recellularization, bioreactors have been developed that can provide perfusion, monitoring, and organ-specific stimuli such as mechanical stretch, pressure gradients, or electric impulses (reviewed in Scaritt et al. [100]). These bioreactors permit the ex vivo culture of whole-organ scaffolds seeded with cells such as primary parenchymal cells, endothelial cells, or stem/progenitor cells. Bioengineered rodent organs that have been generated using this bioreactor-assisted recellularization method have been orthotopically implanted by several groups. These bioengineered organs were able to participate in organ function to a limited extent, but hemorrhage and/or edema occurred within the bioengineered organ as a result of incomplete recellularization [95,98]. Regardless of the challenges ahead, the promise and progress of whole-organ engineering have prompted companies such as United Therapeutics Corporation and Miromatrix Medical Incorporated to invest in developing whole-organ decellularization-recellularization technology toward clinical application using porcine organ scaffolds.

CLINICAL AND COMMERCIAL APPLICATIONS

As the science behind the generation and characterization of ECM scaffolds have expanded, the applications for these scaffolds have exponentially increased. Most commercially available ECM scaffolds are used as a surgical mesh for soft tissue reconstruction or topical wound healing (Table 35.1). Preclinical research has demonstrated the promise of using ECM scaffolds (and recellularized ECM scaffolds) for cardiac patches, vascular cuffs, heart valve replacement, tracheal reconstruction, and potentially even organ transplantation, among numerous other uses.

Regulatory Considerations for Extracellular Matrix Scaffolds

ECM scaffolds have a long history of commercial and clinical application. Most of these ECM scaffolds have been classified as a medical device by the US Food and Drug Administration (FDA). In 1997, the FDA created the Tissue Reference Group (TRG) to assist in categorizing products as a device, biologic, or combination, specifically in the realm of regulating human cells, tissues, and cellular- and tissue-based products (HCT/P). The TRG is composed of representatives from the FDA's Center for Biologics Evaluation and Research, the Center for Devices and Radiological Health, the Office of Combination Products, and the Office of the Chief Counsel. The TRG continues to update recommendations on the classification of products. As of this writing, the TRG indicated that secreted or extracted human products such as collagen are not considered an HCT/P (http://www.fda.gov/biologicsbloodvaccines/tissuetissueproducts/regulationoftissues/ucm152857.htm). In 2014, ground, defatted, decellularized adipose tissue was determined not to be an HCT/P because it is more than minimally manipulated. As a final example, in 2012, allogeneic, processed acellular dermis for breast tissue defects was determined not to be an HCT/P because it is used in a nonhomologous site. It is apparent that FDA regulation of commercial or clinical products is changing as the field of tissue engineering and regenerative medicine advances. For the present, it is hoped that new ECM scaffold-based products will be classified based on their predicates.

CONCLUSIONS

Biologic scaffold materials represent one facet of the multiple strategies used in regenerative medicine to construct functional tissue. Biologic scaffolds are derived from mammalian tissues and thus contain countless structural and functional moieties that have been shown to be necessary for tissue development, homeostasis, and response to injury. Such scaffold materials are currently used in clinical medicine and their use will likely expand as regenerative medicine strategies evolve.

List of Acronyms and Abbreviations

b-FGF Basic fibroblast growth factor
DNA Deoxyribonucleic acid
ECM Extracellular matrix
FACITs Fibril-associated collagens with interrupted triple helices

FDA Food and Drug Administration
GAGs Glycosaminoglycan
HA Hyaluronic acid
HCT/P Human cells, tissues, and cellular- and tissue-based products
MACITs Membrane-associated collagens with interrupted triple helices
MBV Matrix-bound vesicle
PLGA poly(D,L-lactide-co-glycolide)
RGD Arginine-glycine-aspartate
SIS-ECM Porcine small intestinal submucosa extracellular matrix
TGF-b Transforming growth factor-b
TRG Tissue Reference Group
UBM-ECM Porcine urinary bladder matrix extracellular matrix
VEGF Vascular endothelial growth factor

Glossary

Decellularization The process by which cells are removed from a tissue or organ to isolate the extracellular matrix scaffold of the tissue or organ for use in engineering or regenerating new tissue
Recellularization The process by which cells are delivered to and cultured within a tissue or organ extracellular matrix scaffold
Xenogeneic Relating to tissues, cells, or materials belonging to different species.

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CHAPTER

36

Hydrogels in Regenerative Medicine

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INTRODUCTION

To discuss the role of hydrogels in regenerative medicine without extraneous and irrelevant information, it is necessary to set out the meaning and boundaries of regenerative medicine itself. The author of the current described regenerative medicine in 2014, especially in the context of the use of biomaterials in medical technologies [1]. Regenerative medicine addresses a major nonpharmacological, approach to treat disease and injury through the de novo development of functional tissue rather than the replacement of tissue through synthetic devices. Although implantable medical devices can give good performance in treating many conditions, they will always be limited to situations that involve mechanical or physical functions and will not in themselves be able to provide biological solutions to replacing tissue structure and function. Thus, the techniques of regenerative medicine rely on tools that result in regenerating the patient's own tissue; regenerative medicine involves alternative therapies to treat disease and injury by the regeneration of functional tissues or organs instead of replacement by medical devices, transplantation of viable structures or palliative care through the use of pharmaceuticals.

As depicted in Fig. 36.1, there are three main strands of regenerative medicine. The first, usually called cell therapy, involves using groups of cells derived from the patient or elsewhere, which can be injected or otherwise placed at the site of disease or injury in the expectation that they will facilitate the spontaneous regeneration of the required tissue. At its conceptually simplest level, cell therapy does not involve conventional biomaterials, but there are situations in which they may have a supportive role; as will be shown, hydrogels feature prominently here. The second strand is that of gene therapy, in which specific genes are inserted into specific cells to correct deficiencies in those cells, enabling certain processes in tissue expression. It is possible for some biomaterials to be involved in the delivery process, and gene and cell therapies may be combined. The third strand is that of tissue engineering, "the creation of new tissue by the deliberate and controlled stimulation of selected target cells through a systematic combination of molecular and mechanical signals" [2]. Molecular and mechanical signals do not directly imply that the use of biomaterials and tissue engineering may theoretically be carried out in the absence of biomaterials. However, three factors determine that biomaterials are most likely to be involved in tissue engineering processes. The first is that new tissue generated in this way usually needs form and structure, and in themselves, injected cells are unlikely to provide this without the assistance of biomaterials. Second, molecular signals are easily delivered with the appropriate spatial and temporal characteristics; a biomaterial that contains and delivers such signals to the required cells would be beneficial. Third, mechanical signals may be equally difficult to deliver without the sustained effects of a biomaterial support.

As implied earlier, the tissue engineering, cell therapy, and gene therapy modes have some overlapping features; it is not the intention here to become involved in the semantics of these terms or the details of interfaces between them. Instead, this chapter focuses on the role of hydrogels as the preferred exemplars of biomaterials that may be used to support the mechanisms of regenerative medicine and specifically facilitate the delivery of the mechanical and molecular signals, and to assist in generating new functional tissues with appropriate morphological characteristics.

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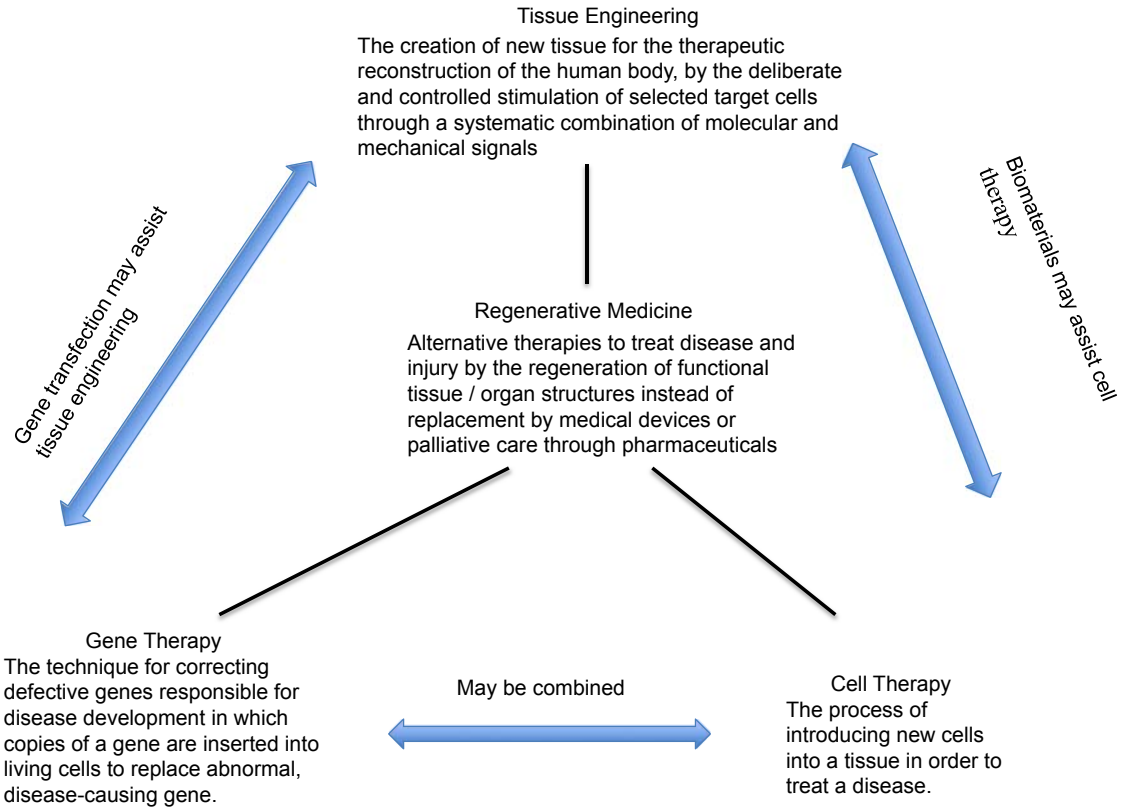

FIGURE 36.1 The essence of regenerative medicine and its components.

BIOMATERIALS TEMPLATES

Nevertheless, it is necessary to briefly address the principles underlying the specifications for the biomaterials that can support regenerative medicine. Specifically focusing on tissue engineering, it has been common practice to describe these material constructs as scaffolds [3]. Conventional scaffolds tend to be composed of discrete porous constructs, usually of polymers or ceramics, in which appropriate cells infiltrate the pores and are intended to express new tissue within these spaces, with the biomaterial degrading and resorbing at the same time. Such constructs have usually been produced by three-dimensional (3D) techniques such as solid free-form fabrication, electrospinning, and solvent casting with porogen leaching. Typical porous scaffolds are seen in Fig. 36.2.

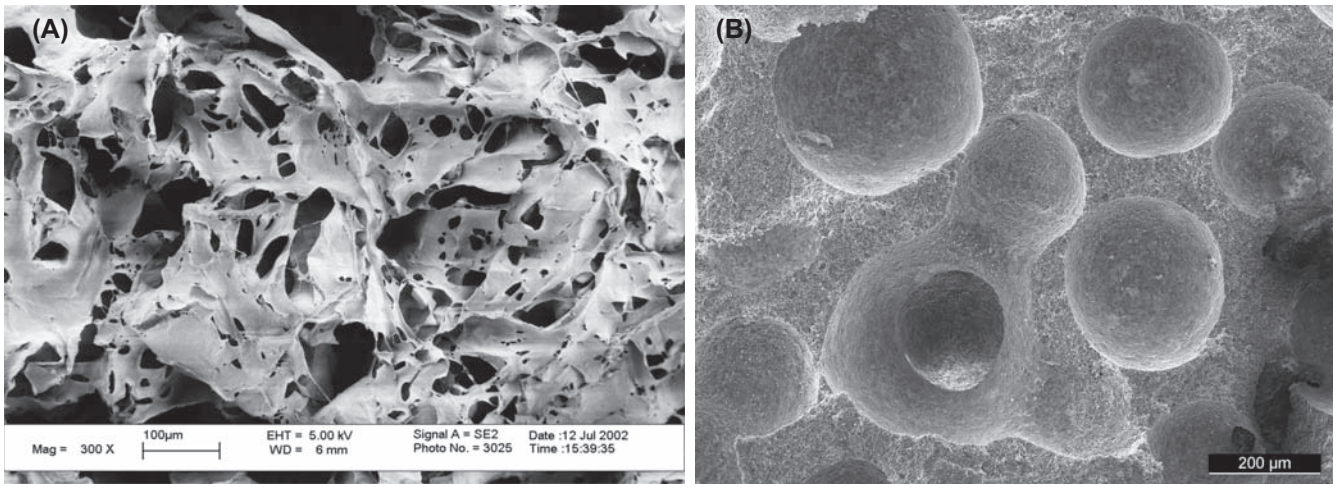

FIGURE 36.2 Examples of conventional porous solid polymer tissue engineering templates.

As discussed by Edalat et al. [4], a scaffold is required to provide an environment, or niche, that favors the natural behavior of cells. The in vivo microenvironment of a cell in general is composed of the relevant extracellular matrix (ECM), homotypic or heterotypic cells surrounding that cell, and cytokines and other bioactive agents around the cells associated with endocrine, autocrine, and paracrine secretions. The microenvironment also involves topographical and architectural features and mechanical forces. It immediately becomes obvious that the type of porous scaffold epitomized by Fig. 36.2 will have considerable difficulty in replicating this type of microenvironment.

This becomes even more significant when stem cells are involved, where the microenvironment is referred to as the stem cell niche. Fig. 36.3, reproduced from Scadden [5], indicates the factors that participate in regulating the system of a stem cell in its tissue state. These include "the constraints of the architectural space, physical engagement of the cell membrane with tethering molecules on neighboring cells or surfaces, signaling interactions at the interface of stem cells and niche or descendent cells, paracrine and endocrine signals from local or distant sources, neural input and metabolic products of tissue activity."

It is suggested here that conventional porous scaffolds do not represent the ideal format for a so-called tissue engineering "scaffold." It is further suggested that, as discussed by the author, the term "scaffold" falls far short of the characterization and specification required for these supporting constructs. The overarching term "template" is preferable [6].

To determine what type of template offers better chances of success than conventional porous solids, it is necessary to define the specifications of these constructs; this has rarely been done within tissue engineering. Edalat et al. [4] have addressed this issue, stating that the engineering of what they describe as scaffolds requires close attention to the 3D microgeometry of the construct (including porosity, pore size, and interpore connectivity), mechanical parameters such as linearity or nonlinearity, elasticity, viscoelasticity, or anisotropy and the successful delivery of biologics including cells, nucleic acids, and cytokines.

These generic requirements can be translated into detailed specifications, some of which are mandatory if optimal performance is to be achieved, whereas others are optional, depending on the precise application [1].

The mandatory specifications are:

- The material should be capable of recapitulating the architecture of the niche of the target cells;
- Because the cell niche is changeable over time, the material should be capable of adapting to the constantly changing microenvironment;
- The material should have elastic properties, particularly stiffness, that favor mechanical signaling to the target cells to optimize differentiation, proliferation, and gene expression;
- The material should have optimal surface or interfacial energy characteristics to facilitate cell adhesion and function;

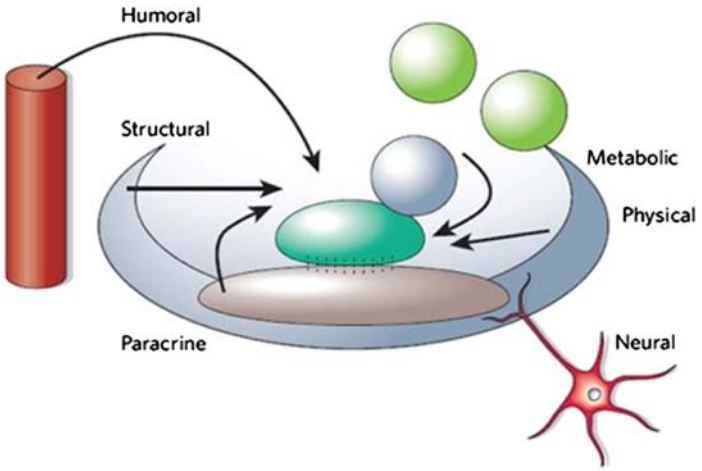

FIGURE 36.3 Components of the local environment that participate in regulating stem cells in their tissue environment. These include the constraints of the architectural space, the physical engagement of the cell membrane with tethering molecules in neighboring cells, signaling interactions at the interface of stem cells, and the niche paracrine and endocrine signals, neural input, and metabolic products. *Reproduced from Scadden DT. The stem cell niche as an entity of action. Nature 2006;44:1075–78, with permission of the Nature Publishing Group.*

- The material should be capable of orchestrating molecular signaling to the target cells by directing endogenous molecules or delivering exogenous molecules;
- The material should be of a physical form that provides appropriate shape and size to the regenerated tissue;
- The material should be capable of forming into an architecture that optimizes cell, nutrient, gas, and biomolecule transport ex vivo, in vivo, or both, and facilitates blood vessel and nerve development;
- The material should be intrinsically noncytotoxic and nonimmunogenic, and minimally proinflammatory.

Optional specifications that vary with the application are:

- The material should be degradable if that is desired, with appropriate degradation kinetics and appropriate morphological and chemical degradation profiles;
- The material should be injectable if that is desired, with the appropriate rheological characteristics and transformation mechanisms and kinetics;
- Where necessary, the material should be compatible with the processing techniques that simultaneously pattern both the material and living cells;
- Where multiple cell types are involved, the material properties should be fine-tunable to accommodate variable cellular requirements, with spatiotemporal control as appropriate;
- When used in a significantly stressed in vivo environment, the material must have sufficient strength and toughness;
- In situations in which the biomaterial encapsulates cells, optimal diffusion characteristics concerning critical molecules are required.

If porous solids are generically unable to comply with all of the mandatory specifications concerned with the cellular microenvironment, consideration has to be given to the type of material that is able to do so; universally the solution to this problem has involved the group of materials known as hydrogels, which are 3D networks composed of cross-linked hydrophilic polymer chains that contain large amounts of water [7–10].

In consideration of these specifications, it is necessary to focus on the characteristics of the composition, structure, and properties of the various forms of ECM [7]. In its natural form, an ECM is composed of water and a mixture of glycosaminoglycans (GAGs) and fibrous proteins, which collectively self-assemble into nanofibrillar supramolecular networks. The precise composition and specific structure vary from tissue to tissue, which implies that the hydrogel for any one construct will depend on the intended tissue application. The main fibrous proteins are the collagens, elastin, fibronectin, and laminin, whereas principal GAGs are hyaluronates, dermatan sulfates, heparin and heparan sulfates, chondroitin sulfates, and keratan sulfates. In addition, many of the GAGs link to core proteins to form proteoglycans. It should be of unsurprising that many of the currently favored hydrogels are based on naturally occurring molecules found in these ECMs. The water content also varies, both with the type of tissue and regionally within any tissue type. Articular cartilage water content varies from 65% to 80% on a regional basis. Within the intervertebral disk, the ECM water content is around 75% in the annulus fibrosis and 80% in the nucleus pulposus; importantly, this decreases with disk degeneration, typically by 10% in advanced cases [11].

From a purely compositional perspective, the ECM appears to be analogous to a hydrogel, albeit a complex one; this was pointed out clearly by a number of reports [12,13]. Naturally, the presence of cells appropriate for the tissue in question and the presence of mineral phases in tissues such as bone and dentin influence the biological and physical properties. The topology, or architecture, reflects the dynamic, reciprocal relationship between the cells and the molecular microenvironment, and the topology and biochemical composition of the ECM is markedly heterogeneous [14,15]. As a form of hydrogel, various types of ECM would be expected to have gel-like mechanical properties, especially those of viscoelasticity, and indeed this is the case [16]. These characteristics vary, especially the complex shear modulus, both regionally within a tissue and with physiological variables such as aging and disease [17,18].

The ECM not only provides the structural and physical support of cells and their biomechanical stimuli in tissue, it initiates critical biochemical cues that are required for tissue function, including morphogenesis, differentiation, and homeostasis. This has to be seriously considered when designing hydrogel templates for tissue engineering. One critical aspect is adhesion between the cells and their ECM, which is mediated by receptors such as integrins. The ECM also is responsible for morphological organization and physiological function by binding growth factors and interacting with cell surface receptors to generate signal transduction and regulate gene expression [14]. The complexity of tissue function requires the ECM to be a dynamic structure that is continually being remodeled and subjected to a variety of posttranslational modifications. Hydrogels used as tissue engineering templates have to address this multitude of characteristics and properties of the ECM.

STRUCTURE–PROPERTY RELATIONSHIPS IN HYDROGELS

As noted earlier, hydrogels are 3D networks composed of cross-linked hydrophilic polymer chains, which can contain large amounts of water, up to thousands of times their dry weight.

The structure–property relationships that characterize these networks depend on a number of factors relating to their source, the polymer composition, the type and nature of the cross-links, their electrical charge, and water-swelling behavior. Hydrogels may be natural or synthetic, or possibly a blend or composite of the two groups. They may be homopolymers (based on a single species of monomer), copolymers (involving two or more different monomer species, at least one of which is hydrophilic), or interpenetrating networks (containing two independent cross-linked components). The hydrogels may be crystalline, semicrystalline, or amorphous. Different properties are seen, depending on the presence or absence of electrical charge on the chains, which may be neutral, anionic or cationic, amphoteric (with both basic and acidic groups), or zwitterionic (with both anionic and cationic groups) in each repeating unit. One of most important characteristics is the ability to swell and deswell reversibly in water on the basis of environmental stimuli; chemical stimuli that induce this behavior include the pH, solvent composition, and ionic strength, whereas physical stimuli primarily include the temperature and electrical or magnetic fields.

As reviewed by Slaughter et al. [19], cross-linking is characterized by junctions, or tie points, formed by covalent or ionic bonds between polymer chains, or by physical entanglements or weak interactions, such as by hydrogen bonding (Fig. 36.4). The resulting network structure can be quantified through a number of parameters, including the polymer volume fraction in the swollen state, the average molecular weight between cross-links, and the distance between them, which is obviously a measure of the mesh size and porosity. This latter parameter is critical for determining the properties of solute transport in the hydrogel, which is obviously important for compliance with the requirement that a tissue engineering template facilitates the transport of nutrients and other molecules. Diffusion is considered to be the dominant mode of transport in tissue engineering hydrogels. This is influenced by the electrical charge, the mesh size, and environmental factors such as the pH and temperature. In complex biological media in which both the polymer and solute are likely to be ionized, interactions between them may have significant effects, generally decreasing solute transport. The precise mechanical characteristics of a hydrogel range from rapid elastic recovery from applied stress or strain to time-dependent viscous recovery; a major factor in tissue engineering hydrogels is the relationship between the glass transition temperature, T_g, and body temperature [19]. At temperatures below T_g there is a tendency for a greater viscous component, with contributions from creep and stress relaxation. However, the very high water content associated with these hydrogels tends to suppress T_g, favoring a more rubbery elastic regime.

We may use the arguments of Buwalda et al. regarding the historic development of hydrogels for biomedical applications [20] to capture the increasing sophistication of hydrogel design as attempts have been made to

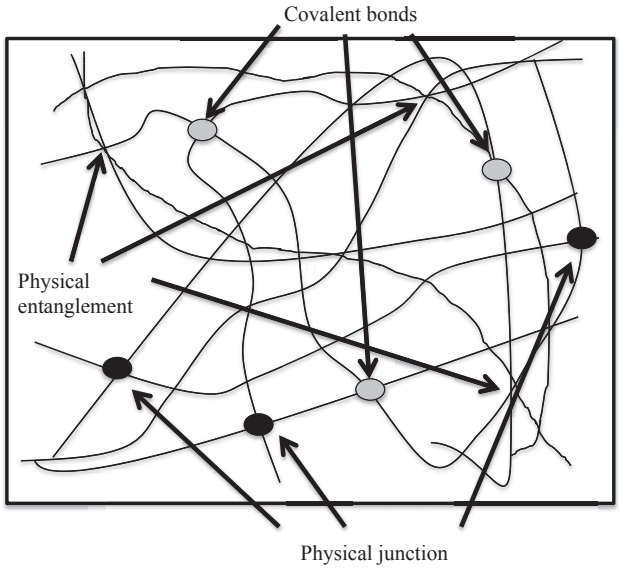

FIGURE 36.4 Hydrogel network with physical junctions, covalent bonds, and physical entanglements.

incorporate the ECM specifications described in the last section. As those authors noted, the first significant event in hydrogel design related to such applications was the introduction of water-swollen, cross-linked, macromolecular networks to construct poly(2-hydroxyethyl methacrylate) (pHEMA) soft contact lenses by Wichterle and Lim in 1960 [21]. The specifications for such a lens were simple and involved sufficient water content for optical transparency, the lack of extractable toxic components, resistance to degradation, good permeability to water-soluble substances (especially oxygen), and suitable mechanical properties; accordingly, the hydrogel structure that was developed was also simple. These first lenses were prepared by the free radical polymerization of 2-hydroxyethyl methacrylate (HEMA) in aqueous solution using ethylene glycol dimethacrylate as a cross-linker.

Buwalda et al. [20] defined this type of simple hydrogel as being a "first-generation" hydrogel. In general, the use of "generation" descriptors is not helpful in biomaterials science [1] because such classifications are subjective, but the use of increasing complexity with specifically designed hydrogels as a marker of suitability for tissue engineering templates serves a good purpose. The baseline of simple synthetic hydrogels encompasses a small group of hydrogels prepared by the polymerization of water-soluble monomers and a group based on cross-linking water-soluble polymers. pHEMA belongs to the first group. These polymers were successful in a number of applications but were soon shown to have several limitations, especially insufficient oxygen (and other solute) transport and mechanical fragility. *N*-Vinylpyrrolidone (nVP) was used as an alternative water-soluble monomer; polyvinylpyrrolidone had higher hydrophilicity and overall improved biocompatibility. Nevertheless, these were not ideal, and various copolymers of HEMA, such as nVP, substituted acrylamides, vinyl acetate, and substituted methacrylates, were subsequently developed. These produced hydrogels with variations in basic properties but with no significant change in the underlying philosophy.

The second group of simple hydrogels, based on the cross-linking of water-soluble synthetic polymers, largely involved poly(vinyl alcohol) (PVA) and poly(ethylene glycol) (PEG). PVA is a linear polymer produced by the free radical polymerization of vinyl acetate, followed by the hydrolysis of the acetate groups to alcohol groups [22]. Cross-linking has been achieved through the use of bifunctional or multifunctional agents that are able to react with the hydroxyl groups of the PVA molecules, including aldehydes such as glutaraldehyde, or through the use of electron beam or γ-irradiation. Obtaining the optimal balance of biocompatibility (potentially influenced by residual chemical cross-linking agents) and mechanical properties has not been straightforward. PEG and its higher–molecular weight counterpart, poly(ethylene oxide), are widely used hydrogels in pharmaceutical preparations; the highly water-soluble polymers are readily cross-linked by γ or electron beam radiation [23]. PEG hydrogels are versatile because molecular chains can have a variety of forms, such as linear or star-shaped, and because they can be biologically modified readily. This provides a good example of how the basic synthetic hydrogels have led to more sophisticated structures that can approximate the ECM paradigm discussed previously.

INCREASING SOPHISTICATION OF SYNTHETIC HYDROGELS FOR TISSUE ENGINEERING

Bioactive Forms of Poly(ethylene Glycol) as Exemplars of Increasing Sophistication

The PEG hydrogels discussed in the previous section provide a good example of how modifications, especially biological ones, can improve the performance of synthetic hydrogels [24,25]. The major limitation of these hydrogels with respect to regenerative medicine applications is the lack of cell-specific adhesion. One approach to overcoming this involves the use of cell-adhesive peptides in modifying the PEG structure. These have generally been derived from the major ECM proteins fibronectin, laminin, and collagen. The most widely used peptide has been arginine-glycine-aspartate (RGD) [26], which can be derived from the cell-binding domain of all three proteins; both linear and cyclic RGD have been employed, with preference for the latter in view of the greater affinity for the integrin $\alpha_v\beta_3$. The peptide may be linked to the PEG network by a number of different groups, such as monoacrylates.

A second modification to PEG involves incorporating growth factors. These are polypeptides that transmit signals to modulate cellular activity, which is important in tissue engineering processes. However, in their free form they have short half-lives because they are susceptible to proteolytic degradation. Growth factors may be incorporated into PEG directly during hydrogel formation, but this usually results in a burst release; this is likely to be unsatisfactory because the dosage response required to facilitate tissue formation is precise and sensitive. Several strategies have been used to address these difficulties, particularly using functional groups to modify the growth factors before tethering them to the PEG network, such as by using cysteine to tether recombinant vascular endothelial growth factor using multiarm PEG vinyl sulfone [27]. Among other growth factors that have been attached to PEG are basic

fibroblast growth factor, transforming growth factor-β, epidermal growth factor, and various bone morphogenetic proteins. Various methods have also been used to mimic the growth factor binding mechanism of GAGs in the natural ECM by the chemical modification of heparin, chondroitin sulfate, and hyaluronic acid, using reactive groups such as acrylate or thiol, and subsequent reaction with functionalized PEG derivatives [28].

With these PEG-based examples in mind, generic forms of hydrogel sophistication are now discussed.

Spatial Heterogeneity

As alluded to earlier, biological processes are usually regulated by spatially dependent signals, in which gradients of molecules are able to regulate cell migration, axon extension, angiogenesis, differentiation, and other processes. As discussed by Khademhosseini et al., control of the spatial location of molecules on a surface and/or throughout a material template could be beneficial for tissue engineering [29]. One approach to generating these gradients involves releasing molecules from a source over time to form a concentration gradient as the molecule diffuses away from the source; in general, however, these gradients are unstable and it is difficult to control their shape. An alternative concept that involves conjugating biomolecules to materials has been used to increase the stability of signaling molecules in a spatially controlled manner. For example, multicomponent, spatially patterned, photocross-linkable hydrogels may be fabricated to localize growth factors within hydrogels. In addition, microfabrication approaches provide attractive technologies because of their availability and cost-effectiveness. The ability to pattern fluids within microchannels has been merged with photopolymerization chemistry to form spatially oriented hydrogels. Hydrogels may be synthesized with gradients of signaling or adhesive molecules or with varying cross-linking densities across the material to direct cell behavior such as migration, adhesion, and differentiation.

These concepts of controlling the microstructure and spatial compositional character in relation to the connectivity between multicomponent tissues, especially the ECM, and hydrogels was discussed by several authors [7,10,12,30–32]. Burdick and Murphy [32] referred to the motivation to introduce spatial heterogeneity into hydrogels, and discussed the role of micropatterning (Fig. 36.5). They claimed that introducing spatially specific cues in hydrogels makes multicellular constructs possible through cocultures or multilineage differentiation.

One widely used micropatterning technique is photolithography, in which a hydrogel precursor material is exposed to ultraviolet light through a photomask that displays the required pattern. This provides reliable shape definition and is able to pattern multiple cells with materials to facilitate the selective adhesion of individual cell types to specific regions; photocross-linkable hydrogels are placed underneath the mask that controls the exposure to generate the structures in the shape of the mask. Soft lithography allows microfabrication at the micron scale, especially using silicon-based elastomers (e.g., polydimethylsiloxane) in microfluidic systems. This is of great potential interest in the development of constructs that have microchannels that resemble the vascular systems of tissues; so far, nutrient and metabolite diffusion has been observed only in relatively small hydrogel-based constructs owing to transport limitations, so microfluidic and nanofluidic techniques that allow for the creation of channels to overcome these limitations are immensely important [33–35].

Most techniques that have been investigated with respect to spatially heterogeneous hydrogels involve nanofibrous architecture [31]; these techniques include electrospinning, phase separation, and self-assembly. Electrospinning is an old technique; it dates back to the 1970s with respect to medical technology, but it is now considerably more sophisticated in relation to the materials used and the structures produced. For example, Sun et al. investigated the use of electrospun photocross-linkable hydrogel fibrous constructs for skin flap regeneration that possess the dual properties of a fibrous nanostructure and hydrogel softness, designed to allow cell migration into the scaffolds to develop 3D microvascular structures [36]. They hypothesized that such a hydrogel would be conducive to endothelial cell adhesion and growth, tubulogenesis, skin flap adhesion of the wound bed, and the formation of microvasculature. This should increase the number of capabilities to aid blood supply and enhance the survival rate of random skin flap after implantation. Gelatin methacryloyl (GelMA) hydrogel, fabricated by incorporating methacrylate groups to the amine-containing side groups of gelatin, was the photocross-linkable hydrogel used. The methacryloyl groups maintained the properties of gelatin and also allowed solidification from liquid to solid permanently via the chemical reaction of the methacryloyl groups. Also, by varying the polymer cross-linking density to control the hydrogel network structure, the mechanical, degradation, and biological properties could easily be fine-tuned. The study demonstrated the suitability of scaffolds for accelerated vascularization and that electrospun GelMA nanofiber scaffolds could support cell adhesion, proliferation, migration in vitro, and the formation of 3D vascular networks in vivo. The photocross-linkable gelatin exhibited controllable mechanical and degradation

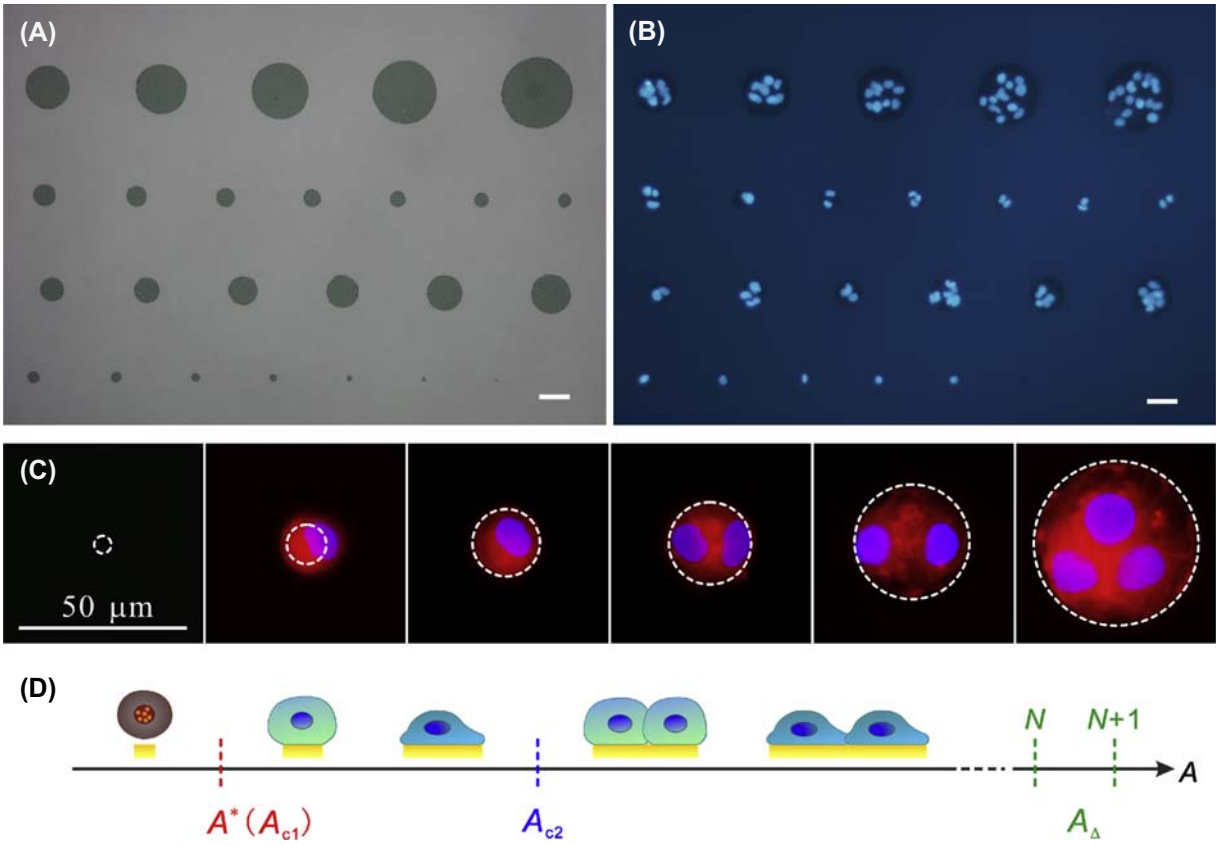

FIGURE 36.5 Cell patterning and critical areas of cell adhesion on micropatterned surfaces with adhesion contrast. (A) Optical micrographs of micropatterns with arginine-glycine-aspartate (RGD) peptide–grafted gold microislands of varying diameters on poly(ethylene glycol) hydrogel. (B) Fluorescence micrograph of MC3T3-E1 cells, a preosteoblast cell line, on a micropatterned surface with cellular nuclei labeled by 4′,6-diamidino-2-phenylindole. (C) Fluorescence micrograph of adherent cells with cellular nuclei labeled in *blue* and F-actin in *red*. *Dashed circles* indicate the contours of the underlying RGD-grafted microislands. (D) Schematic presentation of three characteristic areas for cells adhering on adhesive microislands on a cell-resistant background: A_{c1}, also named A*, the critical area from apoptosis to survival; A_{c2}, the critical area from single-cell adhesion to multicell adhesion; and A_{Δ}, the characteristic area for one more cell to adhere. *Courtesy of Professor Jiandong Ding and Dr. Ce Yan of Fudan University, China.*

properties, resulting in nanofibrous templates to provide the rapid regeneration and formation of cutaneous tissues with minimal inflammation.

Spatial heterogeneity is also important in other tissues and for different reasons, including applications in musculoskeletal engineering [37]. In electrospun constructs, fiber alignment promotes the formation of long lamellipodia extensions parallel to the direction of the fibers, resulting in directional cell orientation and migration through mechanisms of contact guidance similar to that seen in native tissue environmental signaling. Cellular orientation is particularly important in the biomechanics of musculoskeletal tissue, because it determines the pattern of ECM deposition, which is an essential factor in the functionality of bone, tendon, ligament, and cartilage. In bone tissue engineering, for example, fiber alignment and consequent cellular alignment have been shown to regulate cell adhesion and migration, promote osteogenic phenotype, differentiate stem cells toward osteogenic lineage, and enhance mineralization and osteogenesis. Fiber alignment associated with electrospun fibers also closely resembles that seen with collagen fibrils in tendon tissue. Such anisotropy promotes elongated physiological cell morphology, the phenotype maintenance of tendon-derived cells, and the transdifferentiation of other cell types toward tenogenic lineage. The porous 3D nature of electrospun materials also provides a good environment for chondrogenic phenotype maintenance, the chondrogenic differentiation of stem cells, and new tissue formation in vivo in both cartilage and osteochondral defects. Fiber size as well as alignment is an important variable in maintaining cell phenotype and function in cartilage and tendon engineering. One interesting strategy to enhancing the complexity of constructs is based on the use of emulsions or multiaxial nozzles to produce multicomponent core–sheath fibers with multiple, often immiscible components. Such systems have been used extensively for bone, cartilage, and tendon repair.

Electrospinning has also been combined successfully with other fabrication technologies with optimal outputs for various clinical targets. For example, multiphasic scaffolds can be fabricated using electrospinning and additive manufacturing techniques, yielding constructs with large size pores essential for cell and mass transportation, together with fibrous components that provide suitable substrates for cell attachment.

Molecular self-assembly, a ubiquitous biological process, is defined as an autonomous organization of components into patterns or structure without human intervention, and is identified across length scales ranging from DNA base pairing to microtubule fabrication, and up to macroscale tissue morphogenesis [31,37]; unsurprisingly, self-assembly features powerfully in the concepts of structural heterogeneity for the creation of hydrogel-based ECM mimics. The natural extracellular environment is synthesized and organized through self-assembly in hierarchical motifs in which, in a dynamic equilibrium, the structural and chemical milieu needed to promote a range of physiological functions are controlled, including cell morphology, proliferation, attachment, migration, and tissue morphogenesis. Self-assembled template fabrication aims to replicate the sophistication of nature to produce hierarchical 3D tissue equivalents, in which hydrogels have a dominant role. Molecules that are structurally conformable and have chemical complementarity can spontaneously self-assemble into supramolecular architectures under appropriate conditions of temperature, pH, and anionic strength. These will be held together by reversible, noncovalent bonds, which, although they are inherently weak individually, yield strong and stable complexes through their collective interactions. Self-assembled natural, synthetic, or peptide-derived hydrogels have the ability to capture and deliver living cells while controlling their fate. They may also immobilize and control the release of potent biological and bioactive molecules, preserving their molecular conformation and bioactivity. By way of example, collagen nanotextured microfibers, produced by extrusion into a series of neutral phosphate buffers and cross-linking or functionalization solutions at 37°C, represent a significant advance in the recapitulation of the hierarchical architectural organization of musculoskeletal tissues.

The self-assembly of peptides can result in the formation of nanofibers with very high aspect ratios, which may be able to mimic the physical microenvironment of cells, such as wrapping around cells and acting as ties between adjacent structures. One prominent area involves peptide amphiphiles. The development of tissue-engineered nerve conduits used in the setting of complex nerve injury has seen interesting developments with peptide amphiphile nanofibrous constructs. These should mimic the aligned architecture of native nerves to support directional axonal regeneration, but also provide the bioactivity found in the native ECM that facilitates Schwann cell attachment, proliferation, migration, and function. Stupp and colleagues used a series of aligned nanofiber gels formed by self-assembling peptide amphiphiles in peripheral nerve regeneration [38]. Because of their molecular design, these nanofibers can mimic the internal fascicular architecture of peripheral nerves, allowing for the incorporation of Schwann cells vital for peripheral nerve and inducing cellular and neurite alignment and guiding cell migration. They can also be engineered to possess bioactivity that is relevant to nerve regeneration; a peptide presenting the amino acid sequence IKVAV (derived from laminin) was shown to induce neural stem cell differentiation, stimulate neurite outgrowth, and result in functional improvement in acute spinal cord injury.

Matrix Mechanics

The biophysical properties of the ECM are important determinants of many biological processes. As discussed by Gattazzo et al. [39], every cell in its anatomical location has to balance external forces dictated by the mechanical properties of its environment resulting from compression exerted by neighboring cells and the stiffness of the surrounding ECM. The cells have to regulate their own cytoskeleton, generating internal forces that are transmitted to the environment by adhesion sites. These focal adhesion complexes include integrins and signaling proteins, and these physically link the cytoskeleton to the ECM. Mechanical forces are exerted on and by each cell, and this interplay generates a tension within the cytoskeleton that allows maintenance of cell shape and the dynamic response to external forces. This response to mechanical stimuli is referred to as mechanotransduction.

The current author explained elsewhere the pivotal role that mechanotransduction has in many biocompatibility phenomena [40]. Included here are interactions between hydrogels and other template materials with stem cells, as discussed a few years ago by Lutolf et al. [41]. Mechanotransduction profoundly affects the behavior of stem cells, both under natural circumstances and within tissue engineering systems, such as in in vitro bioreactors. The force-dependent cell signaling processes in stem cell differentiation were reviewed by Yim and Sheetz [42], with a special emphasis on focal adhesions, mechanosensitive ion channels, cytoskeletal contractility, Rho guanosine triphosphatase (GTPase) signaling, calcium signaling, and nuclear regulation. Many individual components of the various

pathways in these systems are clearly force-dependent, including the binding of vinculin to talin during initial stages of focal adhesion assembly [43] and the activation of RhoA and Cdc42 in neurogenesis in neural stem cells [44].

Dealing first with in vitro bioreactor-based tissue engineering, two separate types of mechanical cue influence stem cell behavior, only one of which is concerned with a biomaterial property. These aspects were discussed by Steward and Kelly with respect to the mechanical regulation of mesenchymal stem cell (MSC) differentiation [45]. The first type refers to the shear stress system imposed by the mechanics of the bioreactor, which include spinner flasks, rotating wall bioreactors, and perfusion bioreactors, as described by Yeatts et al. [46]. Each of these provides different stress systems and dynamic variations in shear stresses. A primary shear stress–driven signaling pathway in the differentiation of MSCs in both osteogenesis and chondrogenesis is that of mitogen-activated protein kinases. Mechanical stresses are involved in pathway activation and in the upregulation of the proteins on which the pathways depend. Although the physical characteristics of any biomaterial template, including porosity, have some influence on fluid flow, they are not the primary determinant of the shear stresses that affect the cells.

The second type of mechanical cue is that of structural stresses, perhaps best seen in cell-seeded scaffolds in static culture, in which hydrostatic pressure results in stress transfer between biomaterial surfaces and cell membranes. The precise nature of the stresses at these interfaces, including magnitude and type (especially tensile or compressive), has a strong influence on the gene expression of the cells and the differentiation pathway down which they are directed. The mechanisms here are likely to reflect the normal processes of stem cell–matrix interactions within the microenvironment of cell niche; the material property most likely to influence the cell fate is substrate stiffness, or elasticity. In particular, MSCs clearly respond to 3D hydrogel stiffness. They are modulated by integrin binding through the reorganization of ligand presentation at the nanoscale; matrices of 11–30 kPa stiffness induce MSC osteogenic differentiation whereas those of 2.5–5 kPa show adipogenesis.

At this stage, there is a lack of consistency in the details of the causal relationship between stiffness and cell fate when considering all types of cells and all practical conditions, largely because of the interactivity among different mechanisms, but it is clear that mechanotransduction is a primary controlling factor in the phenomena of biomaterial–bioreactor-induced stem cell differentiation. The situation is similar with in vivo tissue engineering, in which much evidence points to a role of mechanical stress in tissue regeneration associated with injectable scaffolds. Myocardial tissue engineering provides a good example. The disparity between the stiffness of myocardium and injectable hydrogels and the importance of associated stress fields were addressed by Reis et al. [47]. When cardiovascular progenitor cells are contained in cardiac ECM–fibrin hybrid scaffolds, their differentiation is affected by the stiffness as well as the composition of the hydrogel. For example, von Willebrand factor gene expression is upregulated with increasing gel stiffness, although such effects vary between adult and neonatal cell sources and with other relevant variables.

The potential role of biomaterials as stem cell regulators was extensively analyzed by Murphy et al. [48]. It is relevant to repeat here a major part of their conclusions: “Although there are many mechanisms at play at the cell/material interface, the fundamental interaction that all cells must have is a link between the cytoskeleton and the material. The consequences of this interaction include a cascade of events in the cell, all of which are initiated by the cytoskeleton or by structures that link it to the material … the cytoskeletal protein actin and its molecular motor myosin II bind and slide past one another to contract the cell. This mechanism is highly organized in muscle, yet it is present in all adherent cell types and in stem cells it enables them to ‘feel’ the stiffness and topography of the environment, as well as to control their size, shape and polarity. Although such inherent properties of the material may seem disparate, they are united by a common contractility-based mechanism that directs stem cells towards specific lineages based on the degree of activation.”

Two further points are worth mentioning. First, some tissues that are prime targets for tissue engineering solutions may be remarkably heterogeneous and/or anisotropic with respect to elasticity, so that replication of the matrix mechanics may not be easy. Within the myocardium, for example, even healthy ventricular ECM can show an effective Young’s modulus ranging from 30 to 75 kPa in different regions; infarcted myocardium can be much stiffer than this [18]. Second, it may be difficult to decouple matrix mechanics from inherent porosity and permeability [49], so that altering hydrophilicity and cross-link density, for example, can have varying effects on stiffness; this has to be taken into account in hydrogel design.

Hydrogel Degradation

It is usually considered necessary for tissue engineering templates to be degradable so that they are replaced as new tissue develops. A few general points need to be made about in vivo polymer degradation in relation to the

mechanisms of degradation and the biological consequences of degradation processes [1]. First, decades of development have shown that it is possible to design biomaterials that are essentially resistant to degradation in tissue environments (such as polytetrafluoroethylene) or are rapidly degraded (such as poly[glycolic acid]) and many that have characteristics in between. The principal mechanism of degradation is hydrolysis, so that susceptibility to degradation is largely controlled by hydrophilicity and the presence of hydrolyzable bonds. By definition, hydrogels are hydrophilic, so their degradation behavior will be determined by the hydrolyzable bonds. If these are numerous and homogeneously distributed, the hydrogel should be rapidly and uniformly degraded. It is also possible under some circumstances for polymers to be degraded by other mechanisms such as oxidation. Polyolefins such as polypropylene may experience oxidative degradation under some in vivo conditions, which is why implantable devices made of such polymers usually contain antioxidants. More important, many biological processes that occur in the vicinity of biomaterials, including inflammation, involve reactive oxygen species, which may become associated with degradation. Other reactions involve tissue enzymes, which may also be able to influence polymer degradation. Because these processes are not necessarily uniform, either spatially or temporally, it follows that degradation effects may be heterogeneous. As noted subsequently, this is an important point in the design of complex heterogeneous or anisotropic hydrogel templates. Second, degradation processes result in the generation of by-products. It is essential that these by-products be compatible with the tissue engineering environment. Ideally, the hydrogel should be bioresorbable: that is, the by-products should be metabolized and harmlessly assimilated into the tissue, such as water and carbon dioxide. It is very important that the by-products not be proinflammatory or cytotoxic. Third, degradation processes may be influenced by mechanical stress, which should be taken into account in template design.

As pointed out by Burdick on several occasions [32,49], uniform hydrogel constructs with homogeneous degradation profiles considerably oversimplify the complexity of the temporal dynamics that are present in tissue development. As implied in the earlier parts of this chapter, the natural ECM undergoes dynamic remodeling through a combination of matrix assembly and degradation, and this characteristic should be replicated in a hydrogel template. These processes occur through the effects of molecules such as proteolytic enzymes, such as matrix metalloproteinases (MMPs), which are produced by cells during migration and signaling. If hydrogels are simple uniform structures in which degradation is solely controlled by cross-link density, there will be no local control over degradation behavior.

In general, hydrogel degradation rates can be fine-tuned by manipulating network connectivity and mesh size. Increased the cross-linking density usually leads to a smaller mesh size, an increased elastic modulus, and slower degradation, because there will be an increased number of cleavable bonds that have to be broken for network mass loss and erosion. Decreased the mesh size also limits accessibility of the degradable moiety to larger molecules, including enzymes, because of a reduced diffusion rate. In addition, encapsulated cells, cell-secreted enzymes, and growth media can influence the degradation rates for chemically or physically cross-linked hydrogels [50]. Hydrogels can degrade by bulk degradation, surface erosion, or a combination of the two (Fig. 36.6). At high cross-link density, restricted diffusion of water and enzymes preferentially leads to surface erosion. In hydrogels with high water content and high diffusivity, bulk degradation occurs when cleavable groups are present throughout the bulk and may degrade simultaneously.

Physically cross-linked hydrogels can degrade by processes that reverse the gelation mechanism or disturb the noncovalent interactions of the cross-links. Chemically cross-linked hydrogels degrade via several mechanisms, including cleavage of the backbone chain, cross-linker, or pendant groups. Also, hydrogels prepared using polymers with degradable functional groups within the backbone chain may be degraded into smaller segments, depending on the location of the degradable groups. Many hydrogels include degradable cross-linkers, such as peptides, proteins, or polymers with chemically labile moieties. These networks degrade into high—molecular weight polymer backbone chains, with degradation products derived from the cross-linker. Polymer chains also can be end-capped with degradable functional groups followed by the addition of reactive functionalities, thus creating cross-linkable degradable macromers. Chemically cross-linked hydrogels may be degraded through hydrolysis, enzymatic cleavage, reversible click reactions, or photolytic degradation. Obviously, to engineer hydrogel degradability, it is necessary to understand the types of cleavable groups and modes of degradation, their by-products, and factors affecting degradation rates.

Polymerization Mechanisms

These sections have referred to the general mechanisms of hydrogel formation and the effects of, for example, different cross-linking processes on the resulting complexity and properties of the hydrogel. The development of

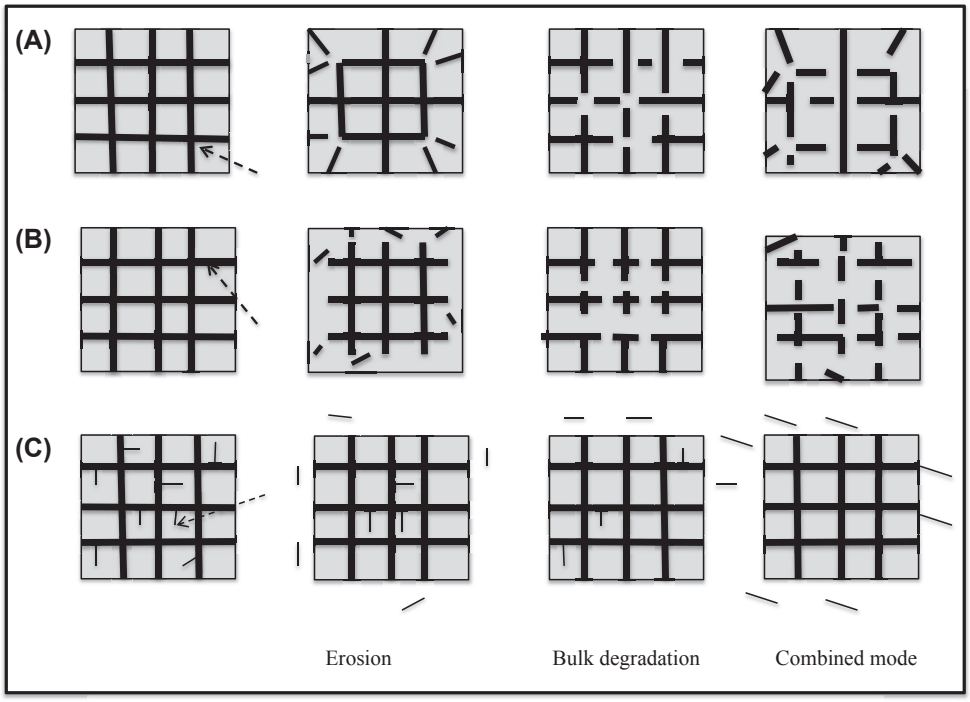

FIGURE 36.6 Degradation mechanisms for hydrogels, involving erosion, bulk degradation, or a combination of these. (A) Polymers subject to degradation at the cross-links. (B) Degradation of the backbone. (C) Release of pendant side groups.

new techniques of cross-link chemistry has led to new structures that are able to mimic the ECM better. Lin discussed such advances with respect to PEG hydrogels [51]; these examples are instructive. It will be recalled that although PEG-based hydrogels have a high water content, tissue-mimicking elasticity, solute permeability, and cytocompatibility, the lack of biological recognition sites is a significant disadvantage. Therefore, PEG hydrogels have been developed that are functionalized through copolymerization with peptides during network cross-linking. For example, acrylated or methacrylate peptides can be copolymerized within PEG-diacrylate or PEG-dimethacrylate hydrogels through chain-growth homopolymerization. PEG-based hydrogels can also be prepared by step-growth photopolymerization, giving a more homogeneous network structure and better mechanical properties. Photopolymerization has the advantage of excellent spatiotemporal control of gelation characteristics. Several bioactive motifs can be incorporated into the hydrogel using this technique.

Click chemistry has been embraced in hydrogel preparation [52] because of the high reactivity and selectivity that can be achieved, as well as the mild conditions that are involved. Click chemistry produces highly efficient, quantitative, orthogonal reactions between mutually reactive functional groups. With reference to PEG, MMP-sensitive peptide sequences can be incorporated into the hydrogels using nucleophilic Michael-type addition reactions between these cross-linkers with terminal cysteines, and multiarm PEG-vinyl sulfone [53]. Cell-adhesive ligands may be readily conjugated using similar Michael-type addition reactions. One major advantage of these types of nucleophilic additions reactions is that they avoid the generation of radicals, which often compromise the biocompatibility of the systems.

Biomimetic hydrogels with good biocompatibility may also be prepared by macromolecular or supramolecular self-assembly, especially where gelation is produced by physical processes, which again avoids the use of radicals. Amphiphilic cyclodextrins are widely used in this context, with physical interactions between their inner hydrophobic cavity and hydrophobic molecules; the hydrophilic outer surfaces facilitate dissolution in physiological environments.

Injectable Systems

In the context of different polymerization mechanisms, mention must be made of the possibility of forming in situ or in vivo gelling systems [54]. The attraction is obvious of being able to inject a viscous sol into the tissue at the site where regeneration is required, under ambient physiological conditions and in a short time (Fig. 36.7).

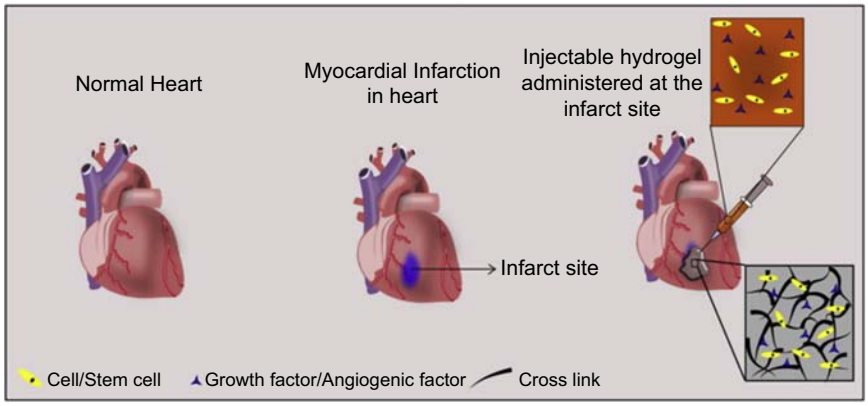

FIGURE 36.7 Example of potential benefits of injectable hydrogels in regenerative medicine. Epicardial delivery of hydrogel-based solution carrying cells and biomolecular signals, which upon administration forms three-dimensional hydrogel over the infarct site owing to cross-linked networks. *Reproduced from Radhakrishnan et al. Biotech Adv 2014;32(2):449–61, with permission of Elsevier.*

In situ–forming hydrogels may be prepared using noncytotoxic cross-linking agents, physical interactions, or self-assembly supramolecular chemistry. These resulting gels should be able to take on the shape and architecture of the site where they are injected and preferably adhere to the local tissue. However, success has been elusive owing to the narrow range of biologically acceptable stimuli for triggering the physical interactions, the generally low mechanical properties, the difficulty of incorporating bioactive agents, and often poor stability.

The range of options for gelling reactions follows the systems discussed in the previous section on general polymerization mechanisms. Much attention has been given to Michael-type addition reactions, especially in PEG, collagen, hyaluronic acid, and heparin systems. Click chemistry is also employed.

NATURAL BIOPOLYMERS AS EXTRACELLULAR MATRIX–ANALOG HYDROGELS

It is not the intention in the next two sections to provide detailed catalogs of tissue engineering hydrogels, but rather to give a summary of the salient features of the various species that are attracting attention. In both categories, i.e., natural and synthetic biomaterials, categories can exist in solid polymer, elastomer, and nanostructured forms as well as hydrogels. Biopolymer or naturally derived hydrogels include those based on hyaluronic acid, alginates, collagen, fibrin, and peptides. They tend to be considered superior to synthetic gels with respect to biocompatibility because they may offer better molecular and morphological cues to cells. However, they have potential disadvantages associated with the sourcing of raw materials from natural origins where purity and consistency may be less than ideal for a quality product used in health care.

Polysaccharides

Generically, a polysaccharide is a complex carbohydrate composed of a chain of monosaccharide units linked by glycosidic groups. Sugars are monosaccharides or disaccharides; polysaccharides have much larger molecules. They clearly have some similarities, but structural differences between different types mean that it is difficult to find common ground that distinguishes the group as a whole from other materials, and they are best dealt with individually. Some of those discussed here are GAGs (mucopolysaccharides), which are long, linear, unbranched polysaccharides of repeating disaccharide units. Some, such as hyaluronan (HA), are nonsulfated, but several, including heparan sulfate, dermatan sulfate, and keratan sulfate, are sulfated.

Hyaluronic Acid

HA, otherwise known as hyaluronic acid, is a linear glycosaminoglycan that has a molecular mass of 10^6–10^7 Da, with very long molecules that consist of linear chains of repeating units of disaccharides of glucuronic acid and *N*-acetylglucosamine.

Two main features of HA have contributed to its attractiveness as a biomaterial [55]. First, it is contained in the properties of many tissues in the human body and contributes to them, which suggests the possibility of

recapitulating some of these within therapeutic products. Among the specific biological properties of HA are its role in embryonic development and wound healing. It is present in high concentrations in synovial fluid and in the ECM of cartilage, so it has a significant role in the functioning of articulating joints. HA interacts with some cell surface receptors and is involved in angiogenesis, cell migration and motility, and tissue organization. It has a role in inflammation and the stimulation of cytokine activity. It can also be functionalized and chemically modified to present a wide range of physical characteristics, with wide-ranging solubility and mechanical properties. The carboxyl group can be functionalized through amidation, esterification, or oxidation. The hydroxyl group may undergo esterification or etherification and the acetyl group may be reacted by deacetylation or amidation.

HA can be derived from a number of sources; one of the most common is rooster comb. It is highly soluble, especially at a low pH, and has a high rate of turnover in human tissue. For the purposes of creating a practical biomaterial, HA is normally cross-linked, for which a number of methods are available, and it may be modified with other substances. Covalent cross-linking provides the opportunity to achieve hydrogels, sponges, and other solid forms while maintaining biological functionality. Cross-linking may take place using water-soluble agents such as a carbodiimide or by the use of photocross-linking using glycidylmethacrylate or methacrylic anhydride.

As a gel, it has high viscoelasticity, a major factor in its use in ophthalmic surgery and in therapies for osteoarthritis. It can be degraded by reactive oxygen intermediates; unmodified HA is rapidly degraded and cleared from the site of administration. To reduce the rate of degradation, cross-links may be introduced. It may also be modified with peptides to enhance cell attachment, spreading, and proliferation. For example, thiol-modified HA can be functionalized with the RGD sequence. These peptide-functionalized gels may also be used as in situ gelling injectable constructs for in vivo tissue engineering.

The areas of tissue engineering most relevant to hyaluronic acid templates are brain and neural regeneration, cardiovascular tissue engineering, skin regeneration, retinal regeneration, and cartilage repair [55]. Several factors have limited the clinical applications so far, including nonspecific protein adsorption and cell adhesion, which can lead to inflammation and the accumulation of degradation products at the site of application, which inhibits stem cell differentiation.

Alginate

Algae are living organisms that are mostly found in water; they can be harvested and provide substances for many industrial uses [56]. These uses are mainly based on their polysaccharide content, although they are often also rich in amino acids such as proline, glycine, and lysine, which accounts for their widespread application as food additives. Seaweeds constitute an important source of harvested algae-based substances because their cell walls contain polysaccharides, which can be readily extracted. Some seaweeds produce agar (from red seaweed); others produce carrageenans. Brown algae (Phaeophyta) produce alginates and several other polysaccharides. The alginates are probably the most important seaweed-derived products and certainly the most significant from a biomaterials perspective. These are harvested in their wild state; cultivation is too expensive, which accounts for some variability in the extracted alginates, because there is some species and seasonal dependence. Alginates are extracted from the seaweed using sodium carbonate and precipitated as either sodium or calcium alginate. This is treated with diluted HCl to produce alginic acid, which us purified and reconstituted into different ionic forms, depending on the application.

Alginates are linear block copolymers of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). The properties of the material will depend on the M:G ratio. Alginates form gels and readily retain water. Different ionic forms have different solubilities; the transition from sol to gel can easily be achieved by conversion between calcium and sodium forms, a process that was extensively used in dentistry to produce elastomeric impression materials. In the solid state, alginates can form films and fibers of good structural quality. When prepared as meshes of nonwoven fibers, they make useful wound dressings, because they are able to absorb large amounts of exudate from the wound bed. Alginate products are used as food additives, moisturizing components of cosmetics, and ingestible preparations for treating inflamed mucosal surfaces. The most attention has been paid in tissue engineering and cell therapies. It is particularly notable that alginates present one of the best options for cell encapsulation, because the hydrated material will allow the diffusion of small molecules essential for metabolic activity but not the immunoglobulins that would attack the cells. The viscosity of alginates and the stiffness after gelling depend on the concentration of the polymer and its molecular weight distribution. Cross-linking between polymer chains can be arranged through multivalent cations (especially calcium) and with carboxylic acid groups in the sugars. Alginates have generally good biocompatibility and can be prepared as an injectable ionic solution. They have poorly controlled degradation and variable cell adhesion characteristics. γ-Irradiation may be used to break high−molecular weight chains to allow faster degradation and clearance in vivo, whereas partial oxidation such as with sodium periodate

increases susceptibility to hydrolysis. There is also interest in derivatization reactions on the polysaccharide backbone that enable, for example, hydroxyapatite nucleation and growth for bone tissue engineering applications and anticoagulation properties through the attachment of heparin [57].

Agarose

Agarose is also a polysaccharide that originates from sea algae; it is composed of repeating units of 3,6-anhydrous-L-galactose and D-galactose [58]. The agarose gels involve a rigid network that is porous and may be used for drug and gene delivery [59]. They can polymerize in situ and are attractive in the context of therapies for central nervous system injury and disease, where they support neurite extension [60]. It is also significant that agarose can form interpenetrating networks with some synthetic hydrogels, such as PEG diacrylate, into which bioactive molecules such as RGD peptides or aggrecan can be covalently immobilized or physically entrapped [61].

Chitin and Its Derivatives

Chitin was first prepared from the cuticle of beetles, from which it derives its name. It is widely distributed in both animals and plants, and is in the shells of crustaceans and mollusks, the backbone of squid, the cell wall of many fungi, within marine diatoms, and so on. Chitin is a linear polysaccharide of β-(1−4)-2-acetamido-2-deoxy-D-glucopyranose, where all the residues are composed entirely of *N*-acetyl-glucosamine: that is, it is fully acetylated. Chitosan is a derivative of chitin, which is a linear polymer of β-(1−4)-2-amino-2-deoxy-D-glucopyranose, in which all of the residues are composed entirely of *N*-glucosamine, which is fully deacetylated. In nature, it is rare for the material to exist as either pure chitin or pure chitosan, and the natural biopolymer will be a copolymer of the two. Generally, when the number of acetamide groups exceeds 50%, the material is referred to as chitin, and the actual percentage is termed the degree of acetylation. Conversely, when the amino groups dominate, the material is referred to as chitosan.

The dry shells of animal sources such as crabs and lobsters contain 20%−40% chitin; the remainder is proteins and calcium carbonate. Demineralization and deproteinization steps are used in the process to prepare raw chitin products. Chitosan can be prepared from the chitin by deacetylation methods involving sodium hydroxide. Moreover, chitosan is able to form a gel by itself without the need of additives. That may happen via hydrogen bonds, hydrophobic interactions, and chitosan crystallites. These hydrogels can also be formed by blending chitosan with other water-soluble nonionic polymers [62] or polyol salts. Because it is polycationic in nature under acidic conditions, chitosan can also form hydrogels through interaction with negatively charged molecules [63]. In addition, the gelation of chitosan could also be obtained through covalent bonding between polymer chains. These bonds make the hydrogel more stable because the gelation is irreversible.

Chitin can exist in three polymorphic states (the α, β, and γ forms); α-chitin is the most common. The biostability varies with the source of the material, the crystallinity, and the degree of acetylation. Chitosan hydrogels are pH-sensitive; they are soluble in dilute aqueous conditions and precipitate into a gel at neutral pH. Generally, chitosan is susceptible to enzymatic degradation. Within animal species, a variety of chitinases are able to break the chitin down into oligosaccharides, which can then be degraded by enzymes such as β-*N*-acetyl-glucosaminidase to yield *N*-acetyl-glucosamine. Similar mechanisms exist for the degradation of chitosan to *N*-glucosamine.

In neuronal repair, chitosan is commonly used to produce tubular structures, such as in the peripheral nervous system [64]. However, chitosan hydrogels have also been applied in neural tissue engineering. For instance, the use of chitosan−glycerophosphate salt hydrogels showed that this type of gel provides a suitable 3D environment for neurons. The addition of peptides such as poly-D-lysine may improve scaffold biocompatibility and nerve cell affinity for chitosan materials (Fig. 36.8).

Cellulose

Cellulose is a linear polymer of β-(1,4)-D-glucose units. It is the main component of the primary cell wall of plants. It forms as crystalline microfibrils that encapsulate the cell with a mesh-like structure, and controls, along with hemicellulose, pectin, and lignin, the mechanical properties of the plants. There is interest in using some derivatives of cellulose as biomaterials in scaffolds and drug delivery systems, for example. Microbial cellulose is a polymer that is synthesized be *Acetobacter xylinum*, a simple gram-negative bacterium. During the synthesis, various carbon compounds are used by the bacteria, polymerized into single linear β-1,4-glucan chains, and then secreted through pores to the cell exterior. These chains then self-assemble into subfibrils and then microfibrils and bundles, which yields a highly 3D crystalline structure with considerable mechanical strength. This nanostructure results in a large surface area that can hold a large amount of water. It can be prepared as a gelatinous membrane that is highly nanoporous. It may be treated with strong bases at elevated temperatures to remove the cells that are embedded in the cellulose

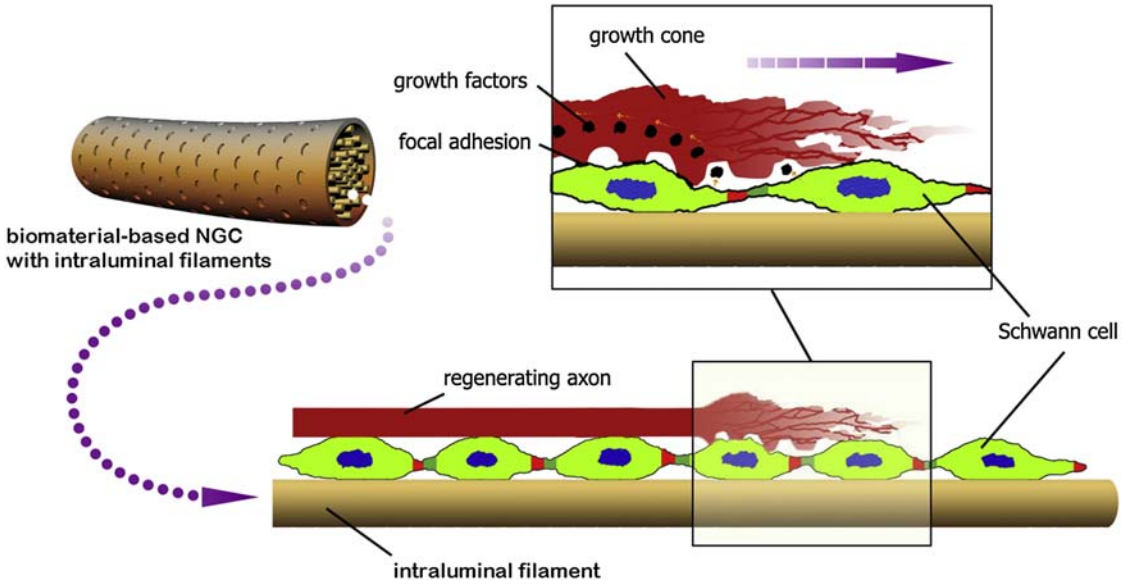

FIGURE 36.8 Schematic diagram of chitosan-based nerve guide conduit (NGC) with intraluminal fillers supporting cell migration of Schwann cells and guiding axonal growth after implantation to bridge a peripheral nerve gap. *Courtesy of Professor Xiaosong Gu, Jiangsu Key Laboratory of Neuroregeneration, Nantong University, China.*

net; it is free of lignin and hemicelluloses so that the final material should be nonpyrogenic, noncytotoxic, and nonimmunogenic. It has shown considerable potential, with support from clinical evidence, for uses in wound healing and possibly as a tissue engineering scaffold. Economic large-scale fermentation systems have been difficult to optimize, which has restricted commercial applications so far.

Proteins and Peptides

Notwithstanding the challenges of purity and consistency mentioned earlier, a wide variety of proteins and their derivatives have been developed as biomaterials, which may be used in several different ways. Most proteins that are used as biomaterials are based on those found in mammalian tissue. These include the structural proteins collagen and elastin, and also some that are derived from plasma proteins, including fibrin and fibrinogen. These structural proteins could be used in what is essentially their natural form: that is, as the mammalian tissues themselves, with varying degrees of processing. Alternatively, they may be extracted from such tissues and subjected to some form of purification and reconstitution. Also, because of the inherent variability in such products, it may be possible to prepare the materials by recombinant technologies. There are some generic and important differences between the reconstituted and recombinant forms of protein biomaterials. In the former case, there is always a risk of contamination with prions and viruses, but this risk is not present with recombinant proteins. The latter are fully characterized, consistent, and reproducible, whereas the former depend on the source quality and batch-to-batch variability may be high. From the perspective of commercial manufacturing, recombinant proteins will usually be expensive but are amenable to proprietary processes that can be protected by patents and trademarks, which is not as readily applicable to the natural products.

Collagen and Its Derivatives

Collagen may be prepared in various forms of gel for tissue engineering applications, including those with the ability to form in situ. Many of these applications involve unmodified collagen; chemical cross-linkers can be used to inhibit degradation and resorption when necessary.

Collagen in one form or another has been used as a biomaterial for many years. Catgut sutures, which are rarely used now, were the mainstay of surgical wound closure for a long time and were made from the collagen of bovine intestines. Purified collagen has been used in injectable form as a tissue filler for both functional (vocal chords and urethra) and cosmetic (facial) effects. Some of these products are xenogeneic; they are derived from bovine dermis, for example, whereas others are allogeneic and are obtained from cultured human dermal fibroblasts or from human cadavers. Formulations vary considerably. Some are simple suspensions (at less than 5%) in buffered saline. Others

are prepared as micronized particles contained in syringes that are hydrated just before use. Depending on the source of the collagen, these products have the potential to be immunogenic. A small number of patients show immunological intolerance. Procedures usually involve prior testing of sensitivity. The collagen filler becomes incorporated into the patient's tissue, but some volume loss will occur over time.

The precise biochemical characteristics of the collagens used in these products are not significant because the material simply acts as a space filler. The applications of collagen in tissue engineering, wound healing, and drug delivery are more dependent on these characteristics. The structural order of collagen occurs at several different levels, and there are many different forms of the protein in mammalian tissue. Of relevance here is the need to balance the mechanical properties and biological activity of the products; this balance will change with the specific type of collagen.

At this stage, collagen types I and III, alone or combined, are most commonly used in products of regenerative medicine. Conduits for peripheral nerve repair are good examples. There are products made from type I or mixed types I and III in clinical use. These have different structures; some conduits are homogeneous fibrillar structure whereas others have heterogeneous structures with concentric cylindrical structures. The materials are processed in ways to minimize antigenicity, such as by cross-linking or the enzymatic removal of antigenic nonhelical telopeptides. Degradation rates vary from a few months to a year or so. In some situations, the collagen may be copolymerized, such as with GAG molecules. Collagen type I is also used in a number of products in bone tissue engineering, although not on its own. It is usually combined with hydroxyapatite or tricalcium phosphate, either as a reinforced composite or as phosphate-coated collagen fibrils.

It was noted earlier that recombinant collagen may have some advantages over animal- or human-derived materials. A number of methods are available for recombinant collagen production. This occurs in bioreactor-based eukaryotic systems, mammalian cell culture, insect cell culture, and many other systems. However, the best results and the formation of collagens most appropriate for human regenerative medicine applications are obtained with mammalian cells transfected with collagen genes, in which hydroxylated full-length collagens are produced. Recombinant human collagen of types I, II, and III can be reconstituted into fibrils that can be processed into forms such as fleeces, 3D gels, and sponges, in which it is anticipated that type I will be used in bone tissue engineering, type II in cartilage, and type III in vascular tissues.

In general, collagen products used in medical technology are structural materials rather than gels [65]. Antoine et al. reviewed the specific features of type I collagen that are associated with its use as a hydrogel in tissue environments [66], especially in relation to the encapsulation of viable cells in the constructs. As alluded to earlier, the properties of collagen biomaterials are variable, depending on a large number of fabrication parameters, and this is especially relevant for collagen hydrogels. The collagen source is particularly influential, with marked differences between animal sources (e.g., murine, porcine, bovine) and tissue types (tendon, skin, etc.) The method by which the collagen is extracted from the tissue is also critical. Acid solubilization is usually used for minimally cross-linked collagens whereas a combination of neutral salt solution with proteolytic digestion is needed for highly cross-linked collagens to denature them fully. The polymerization temperature affects the properties of the resulting hydrogel, with more rapid self-assembly and therefore less ordering as the temperature increases; the temperature used will reflect the need to avoid cell damage. Collagen concentration is also important, although correlations between this and resulting mechanical properties are not clear. As with the temperature, the pH is influential; cell viability will be negatively affected if the pH is outside the range 7.4–8.4, so the better properties that may be achieved at the extremes of pH are not relevant.

Gelatin is obtained by the partial hydrolysis of collagen obtained from bone and other connective tissues of animals. When obtained under acid conditions, it is known as type A gelatin, and under alkaline conditions as type B. It is used in foodstuffs and in many pharmaceutical formulations. It is also used as a gel to provide an initial seal within vascular grafts, in which situation it may also be a drug carrier, such as in the delivery of antibiotics.

Elastin Derivatives

Elastin is the dominant protein of elastic tissue fibers; as such, it is an important component of the ECM of tissues, such as those of the lungs, skin, and blood vessels, which depend on elasticity for their function. It is derived in vivo from cross-linking of the tropoelastin monomer and is essentially insoluble. The tropoelastin/elastin molecules have hydrophobic regions from which the resulting elasticity is derived, and hydrophilic regions, which provide sites for amine-dependent cross-linking and biological signaling. The elastin possesses a number of peptide motifs that are able to influence cell behavior, including proliferation and differentiation. Such interactions take place through several cell-surface receptors, including the elastin-laminin receptor. The signaling of cells in wound healing by elastin controls the relative activity of dermal fibroblasts and contractile myofibroblasts and hence determines the mechanical properties of the subsequent repaired skin.

The insolubility of elastin, which restricts processing, and its relatively poor strength, has limited the practical applications of this protein in biomedical applications. Thus, elastin-like or elastin-based materials have attracted more attention [67]. There are solubilized forms of elastin, including α-elastin, which is obtained under acid conditions, and κ-elastin, which is obtained under alkaline conditions. Recombinant techniques have allowed the preparation of materials that are able to mimic the important and desirable regions of the elastin. Synthetic analogs of elastin may also be prepared from the aqueous processing of replicas of tropoelastin. Many of these substances are amenable to scale-up manufacture, giving a range of elastin-derived products for biomedical uses. For tissue engineering applications, many of these derivatives may be processed by electrospinning or prepared as hydrogel matrices. It is also possible to prepare blends involving elastin derivatives and other proteins, such as silk and collagen.

Desai et al. discussed critical issues with elastin-based hydrogels [68] and noted that many applications requiring large and repeated deformations can benefit from rubber-like elastomeric hydrogels; there are examples in regenerative medicine that come into this category. However, hydrogels generally lack sufficient strength and elastic extensibility owing to cross-link inhomogeneity in the networks. Short, uneven intercross-link distances and poor chain extensibility limit the ability of the hydrogels to stretch. Various strategies have been used to improve hydrogel properties, such as extensibility and toughness through physical bonds. However, time-dependent recovery of physical cross-links in these hydrogels results in high hysteresis and stress softening that can lead to a loss of performance under repeated deformation. Creating a rubber-like elastomeric hydrogel requires control over the architecture of the cross-linked network, which should be homogeneous, and a careful choice of polymer. The chain length between cross-links should be constant to avoid local concentrations of stress, whereas the cross-linking scheme should ensure proper chain incorporation to avoid dangling chains that do not participate in the elastic network. Ideally, the polymer should behave like an entropic spring that recoils once unloaded, in which the driving force restoring an entropic spring is an increase in entropy as the chain goes from a stretched state with limited movements to a coiled state. Protein-based polymers may provide opportunities for engineering hydrogels with these network and polymer properties. To create elastomers, elastin-like polypeptide (ELP) sequences provide a natural choice because they have been characterized as entropic springs. ELPs are built on a repetition of the pentapeptide Val-Pro-Gly-X-Gly with a guest residue "X" that cannot be proline. They are thermoresponsive and undergo the process of inverse temperature transition in which they transition reversibly from a hydrophilic to a hydrophobic state. Thermodynamic factors including the hydrophobic interactions within the chains keep the polypeptides in an unstructured coil form after the transition and have a role in the elastomeric nature of the polypeptides. These elastomeric hydrogels are clearly interesting for tissue engineering applications.

Fibrin Derivatives

Fibrinogen is a soluble protein in blood that is converted to an insoluble fibrin network in the presence of thrombin during coagulation. The fibrinogen has three pairs of polypeptide chains that are joined by disulfide groups. The central domain contains fibrinopeptides A and B, which are cleaved in the presence of thrombin to form the fibrin monomer. These monomeric units form two-stranded fibrils that undergo covalent cross-linking to form the fibrin network, a process that is facilitated by $CaCl_2$. This process can be recapitulated artificially in biomaterial products, generally known as fibrin glues or fibrin sealants, in which separate preparations containing fibrinogen and thrombin are mixed just before application at sites of surgical injury, such as during cardiopulmonary bypass, to assist in sealing tissue defects such as fistulae and facilitate tissue repair as in peripheral nerves.

It is possible for fibrin glue to be derived from autogenous or allogeneic blood, although the latter is more common in commercial products. Blood components are obtained from the blood and undergo various screening and purification procedures, especially when pooled blood is involved. Fibrinogen may be isolated from the blood using centrifugation and cryoprecipitation. Typically, sodium citrate solution is added to the blood to anticoagulate it before centrifugation. The cryoprecipitation procedure may be carried out by a variety of regimes, usually using temperatures between $-20^{\circ}C$ and $-80^{\circ}C$. $CaCl_2$ and aprotinin are added to the solutions to optimize the gelation of the fibrinogen and thrombin solutions once mixed.

The role that fibrin has in response to the injury of vascularized tissue may also be recapitulated in the formation of tissue engineering scaffolds, which have been used in many experimental systems involving several different tissues. Fibrin hydrogels prepared, as discussed earlier, from commercially purified allogeneic fibrinogen and thrombin have some attractive features but are not ideal. Mechanically, they are weak and not stiff, and they have a tendency to degrade quickly. The gels also undergo considerable shrinkage during formation. In addition, the fibrin is not particularly active biologically in this format. Several procedures may be used to modify the gel to obtain better performance. Shrinkage may be minimized by incorporating poly-L-lysine. Using a variety of synthetic polymers or calcium phosphate additions, composite or copolymer scaffolds may improve the mechanical

properties. Cross-linking agents may improve stability. In addition, biologically active peptides or therapeutic proteins can be incorporated into the hydrogels, such as by incorporating functionalized PEG. Najjat et al. showed that it is possible to engineer fibrin gels incorporating proangiogenic growth factors that are able in a murine model to promote the engraftment of pancreatic islets in extrahepatic sites [69].

Silk

Silks are proteins that are synthesized by Lepidoptera larvae such as silkworms and spiders. They are biosynthesized in epithelial cells and secreted into the lumen of specialized glands, where they are stored and subsequently spun into fibers. The properties of silk vary considerably with their source, with different amino acid sequences and mechanical properties that are fine-tuned to their specific function. The most widely used and investigated silks with respect to biomaterials applications are derived from the domesticated silkworm, *Bombyx mori*, and from spiders such as *Nephila clavipes* and *Araneus diadematus*.

Silkworm silk is popular and has been used for medical devices such as sutures for many years; for general textiles, it has been used even longer. This silk has two major fibroin proteins, light (25-kDa) and heavy kDa chains, where the core sequence repeats include alanine-glycine with serine or tyrosine. These core fibers are encased in the glue-like sericin protein. Spider silk proteins range from 70 to 700 kDa; many such silks are characterized by polyalanine and glycine regions. Spider silk is not easy to harvest, and much emphasis has been placed on the use of genetic engineering techniques to produce synthetic versions. Cloning and expression of silks has been achieved in a number of host systems, because the sequences of complementary DNA and genomic clones encoding spider silks show highly repetitive structures that can be used to construct genetically engineered spider silk–like proteins.

Silk fibers have significant hydrophobic regions and high crystallinity with extensive hydrogen bonding, which give good environmental stability and mechanical properties. They are insoluble in most solvents including water. The crystallinity results from the presence of small β -sheets within the fibers. *B. mori* silk can have an ultimate tensile strength of 740 MPa, a Young's modulus of 10 GPa, and a 20% strain at break. Spider silk may have values of 950 MPa, 12 GPa, and 18% respectively.

The biocompatibility of silk products varies, largely because of the varying levels of nonfibroin components such as the sericin. When used as a suture material, silk elicits a greater inflammatory response than do most synthetic polymers. Although it is classified as nonabsorbable, it degrades slowly. Silk fibers losing their tensile strength over a year or so. Proteases such as chymotrypsin can cleave proteins to peptides, especially in amorphous regions. Various silks have found utility in tissue engineering applications because they are often able to support cell growth [70].

As discussed by Melke et al., silk fibroin can be used in several different formats in tissue engineering [71]. Sponges made by freeze-drying or porogen leaching techniques are easily produced and give good porosity, although the precise design of architecture is difficult. Fibers may be prepared by electrospinning, although reproducibility is problematic. 3D printing is used, with controllable geometry and cell encapsulation, although with low resolution. The hydrogel format (Fig. 36.9) is probably the most versatile; there are opportunities for in vivo injectable cell-encapsulation applications, although the small pore size is a disadvantage.

Self-assembled Peptides

An increasingly important class of hydrogels for regenerative medicine is those made from self-assembled peptides [72]. These are polypeptides that assemble under specific conditions to form nanoscale structures. One prominent example is the class of self-assembled peptides made from amphiphilic molecules, derived from polypeptides linked to a polycarbon chain. The polypeptide region is typically hydrophilic whereas the hydrocarbon chain is hydrophobic. They can self-assemble into rod structures because of the arrangement of the hydrophobic regions as well as the charge shielding of the hydrophilic end groups by ionic molecules in the solution. These molecules can be decorated with functional groups to facilitate cellular adhesion and signaling. A number of other self-assembling peptides have been produced with advantages such as the ease in which gels are formed and functionalization. They tend to be mechanically weak.

The significance of these engineered peptide hydrogels is that they epitomize this direction toward materials that can replicate cell niches, referred to earlier as the most important specification for tissue engineering templates. Considering the stem cell niche in particular, these contain ECM components such as laminin and hyaluronan, which present cell-adhesion ligands, and soluble factors such as cytokines and growth factors, with a constantly replenished supply of differentiation cues. Peptide materials can be designed at a molecular level to bestow combined structural and biological activity characteristics that start to address these niche characteristics. These engineered, self-assembled peptides contain relatively short chains of amino acids. Through the careful choice of amino acid monomer sequences, the peptides can fold into secondary structures such as β-sheets, which themselves

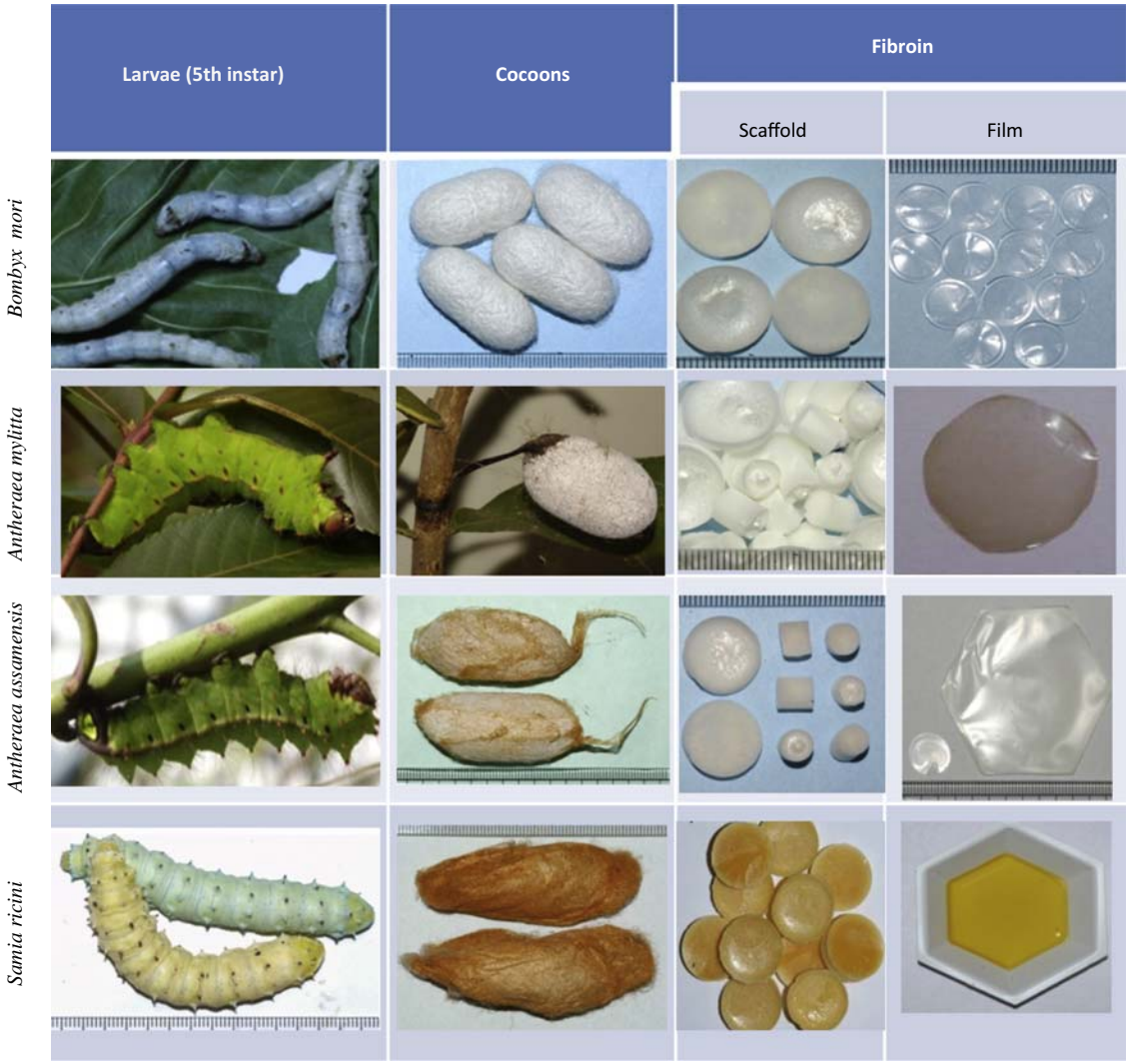

FIGURE 36.9 Different silkworms and silk-derived biomaterials. Sericin can also be fabricated into different biomaterials such as scaffolds, films, hydrogels, nanofibers, and particles. Images courtesy of Professor Kundu, Indian Institute of Technology Kharagpur, India.

self-assemble into hierarchical structures such as fibers and micelles. These fibrous hydrogels replicate the required cell niches far better than do other materials. It is possible that such structures may be reinforced by other nanoscale structures to give robust templates.

Several forms of self-assembled peptides have reached advanced stages of development, with good biocompatibility and degradation properties and without immunogenicity [72]. B-strand peptides, which assemble into discrete β-sheets, appear to be favored. For example, templates consisting of alternating amino acids that contain 50% charged residues may be prepared. The β-sheets have distinct polar and nonpolar surfaces; included here are several self-assembling peptides such as RAD16-I and RAD16-II, in which stable macroscopic matrix structures can be fabricated through the spontaneous self-assembly of aqueous peptide solutions introduced into physiological solutions. Such peptide structures support the cell attachment of a variety of mammalian cells.

SYNTHETIC HYDROGELS FOR TISSUE ENGINEERING TEMPLATES

An informed discussion about the current situation with synthetic hydrogels in tissue engineering is problematic; despite a vast amount of research and development in this area, and although such hydrogels have a reasonably good record in drug delivery systems, there has been little progress in introducing them into clinical tissue engineering products.

One important consideration is that of intrinsic biocompatibility. It is recognized that the chemicals used in preparing hydrogels may have some toxicity, and care has to be taken if the degree of conversion is not 100%; initiators, organic solvents, stabilizers, emulsifiers, unreacted monomers, cross-linking agents, and other substances have to be considered in this light and such chemicals may need to be removed. Synthesis typically should be followed by purification processes such as solvent washing or dialysis. When any of the materials are derived from natural sources, they may carry the risk of batch-to-batch variation, which also has to be taken into account. Synthetic hydrogels have some important advantages over natural biopolymer-based hydrogels, including easier large-scale production and fine-tunable and consistent properties. However, many are made using harsh synthetic chemistry, which requires care to ensure that contaminants and unreacted reagents present during synthesis are then removed. Reaction schemes for these hydrogels often rely on multifunctional cross-linking agents. Free radical polymerization is widely used with many tissue engineering systems that require in situ formation.

The main difficulty, as pointed out several times in this chapter, is that truly synthetic hydrogels usually do not have the biological functionality to provide for the specifications that have been defined for template materials. Thus, many of the newer developments have addressed this major deficiency by introducing functionalization methods and using hybrid or composite structures, often through blending or copolymerization with one of more of the biopolymers discussed earlier. Following the pattern of the Bioactive Forms of Poly(ethylene Glycol) as Exemplars of Increasing Sophisticationsection, the experiences with PEG may be used as an example.

PEG is a diol with two hydroxy end groups; it is formed by linking repeating units of ethylene glycol. The versatility arises from two facts; first, it has linear and branched forms' and second, these end groups can readily be functionalized, such as through carboxyl, amine, thiol, or azide groups. These functional end groups can be symmetric or asymmetric. The latter option is effective because it enables simultaneous but different properties to be achieved. A popular form of PEG is the four-arm-PEG, but other geometries are possible.

The versatility is also facilitated by the possibility of cross-linking by different methods under different conditions. Free radical polymerization, condensation reactions, enzymatic reactions, and click chemistry can all be used, but the most common cross-linking procedure is photopolymerization. This allows the conversion of liquid to solid state under ambient physiological conditions in situ, with good spatial and temporal control and with the possibility of the simultaneous incorporation of biological species or active agents. Best results are normally achieved through the use of acrylates such as diacrylates or dimethacrylates as the macromers. PEG is normally nonbiodegradable and has little intrinsic biological reactivity; a major use of PEG is as a coating to minimize protein adsorption to surfaces. Unmodified PEG is unattractive for tissue engineering applications because of the lack of support for cell function.

PEG itself, in a low−molecular weight, unmodified form, has a number of mundane medical uses, largely as over-the-counter preparations such as laxatives and skin moisturizing agents. The real attraction of PEG as a biomaterial is associated with its combination with other molecules. There are two main scenarios here: either the PEG is used to surface modify a structure to provide or hide properties, or other molecules are used to modify PEG hydrogels to capitalize on the hydrogel characteristics but provide some specific biological activity. The first of these options is referred to as pegylation. There are many examples ranging from pharmaceutical molecules to nanoparticles. It has long been known that pegylation of protein and polypeptide drugs can alter both their pharmacokinetic and pharmacodynamic properties, such as by increasing water solubility, minimizing cytotoxicity, and reducing renal clearance. At its simplest, PEG makes the drug molecule larger; each ethylene glycol subunit is tightly associated with two or three water molecules, which makes the pegylated molecules appear up to 10 times larger than the unmodified molecule. The PEG acts a shield around the molecule, protecting it from degradation and rapid clearance. The effect may be much more subtle, depending on the chemistry involved. For example, the bond between the PEG and the drug molecule may be intentionally unstable to improve targeting, such as when the bond is cleavable by enzymes within the endosomal compartment of cells, which releases the peptide or protein molecule within the cell.

Bioactive modification of PEG hydrogels for tissue engineering applications may facilitate cellular function. A variety of ECM protein-derived cell-adhesive molecules may be incorporated into the hydrogel to increase cell adhesiveness. The hydrogels may be made degradable by incorporating hydrolytically or enzymatically susceptible segments. Growth factors that have been functionalized may be covalently attached to the PEG hydrogels, especially those that involve multithiol or multiacrylate groups.

PEG has been combined with a variety of nonhydrogel biodegradable polymers such as poly(glycolic acid) [73] and reinforced with fibrous collagen [74]. Carbon nanotubes (CNTs) have also been used to create hydrogel composites [75]. CNTs can impart electroconductivity to otherwise insulating materials, improve mechanical stability, guide

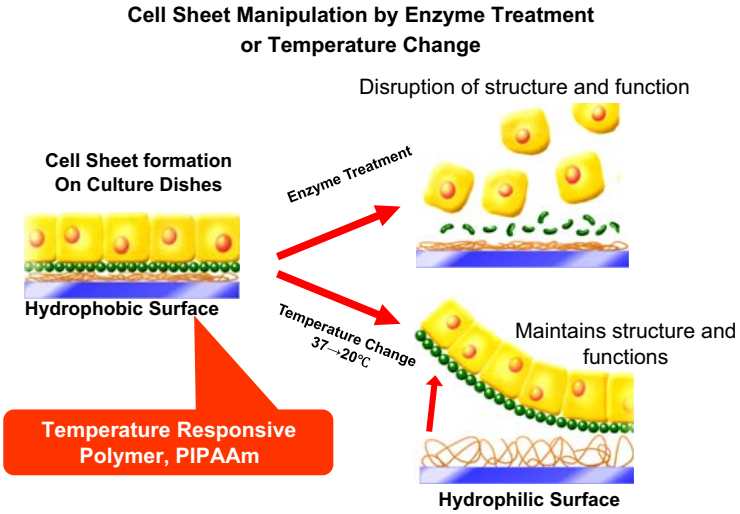

FIGURE 36.10 Principles of cell sheet engineering. Conventional techniques to release cells from biomaterials surfaces using enzymes result in disruption of the cells. Techniques of cell sheet engineering, which involve a change in the hydrophobic–hydrophilic balance of a thermally responsive hydrogel such as poly(*N*-isopropylacrylamide) (PIPAAm), produce the release of sheets of cells without disruption. *Courtesy of Professor Teruo Okano, Institute of Advanced Biomedical Engineering and Science, Tokyo Womens Medical University, Japan.*

neuronal cell behavior, and elicit axon regeneration. A PEG hydrogel composite was prepared in which the CNTs were entrapped in the hydrogel phase during gelation. The hydrogel cross-linking reaction was based on Michael-type addition, which is ideal for in situ cell and protein encapsulation, and in which sonication and surfactants were used to disperse the highly hydrophobic CNTs in the aqueous polymer solution. The inclusion of the CNTs impeded hydrogel crosslinking leading to longer gelation times, higher swelling and porosity, and lower storage modulus above a threshold CNT concentration. Unlike the PEG hydrogel alone, the PEG–CNT hydrogel composite was capable of supporting high neural cell viability in which the CNTs provided sites for cell attachment.

As a final point, mention should be made of thermoresponsive hydrogels, which have potential uses in regenerative medicine. Poly(*N*-isopropylacrylamide) (pNIPAAm) is a good example [76]; it is a polymer with lower critical solution behavior in aqueous solvents and a lower critical solution temperature (LCST) around 32°C. During the phase transition, the polymer chains undergo a change in conformation from an extended coil to a globular structure. The LCST of *N*-isopropylacrylamide (NIPAAm)-based polymers can be increased with copolymerization with more hydrophilic monomers, resulting in a transition temperature close to physiological, which can be useful in medical applications, including drug delivery, tissue engineering, and cell sheet engineering. The homopolymer of NIPAAm is not biodegradable, and many studies have focused on imparting biodegradation by adding various monomers into the polymer structure, such as by copolymerizing NIPAAm with benzomethylene dioxepane.

The development of in situ–forming materials with a dual solidification mechanism, a physical gelation imparted by NIPAAm and a covalent cross-linking reaction, is interesting. The dual gelation mechanism results in hydrogels with significantly enhanced mechanical properties compared with hydrogels solidified only by physical or chemical means. Covalent cross-linking mechanisms include Michael-type addition between thiols and vinyl groups, functionalization of thermogelling macromers with (meth)acrylate groups, cross-linking with a thermal initiator, and epoxy functionalities reacting with amines.

Substrates based on NIPAAm have been also used to fabricate cell sheets. When heated above the transition temperature, the hydrophobic nature of pNIPAAm favors cell attachment. The LCST of the pNIPAAm-based polymer can be fine-tuned to be below cell culture temperatures. When the temperature is lowered below the LCST, the polymer surface becomes more hydrophilic and cells can be harvested in a confluent monolayer (Fig. 36.10). This process eliminates the use of proteolytic enzymes or mechanical means for cell detachment from cell culture surfaces [77].

CONCLUSIONS

Much progress has been made in the development of hydrogels as templates in tissue engineering and regenerative medicine. However, there is a long way to go. The ultimate aim is to create templates that can meet all of the specifications set out in the early parts of this chapter, especially concentrating on replicating all of the characteristics of the ECM. Although hydrogels are superior to porous solid biomaterials in this respect, the architectural and

functional requirements are exacting. Most synthetic hydrogels are too far removed from ECM characteristics for us to have serious expectations for their scientific and clinical success. Natural biopolymers have many advantages, but they still have limitations. Hybrid, composite, and functionalized structures are showing attractive properties in many situations.

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CHAPTER

37

Surface Modification of Biomaterials

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INTRODUCTION

Biomaterial Interfaces in Regenerative Medicine

Biomaterials, whether synthetic (e.g., polymers, metals, ceramics) or natural (e.g., proteins polysaccharides), have central roles in tissue engineering and regenerative medicine applications by providing three-dimensional scaffolds to support cellular activities, matrices for the delivery of therapeutic agents (e.g., drugs, proteins, DNA, small interfering RNA), and functional device components (e.g., mechanical supports, sensing/stimulating elements, nonthrombogenic surfaces, diffusional barriers). The bulk properties of the biomaterial are critical determinants of the biological performance of the material [1]. For example, the mechanical properties of a vascular substitute, including elastic modulus, ultimate tensile stress, and compliance, dictate the ability of this tissue construct to support the applied mechanical loads associated with blood flow. On the other hand, the biological response to a biomaterial is governed by the material surface properties, primarily surface chemistry and structure. Protein adsorption or activation and cell adhesion, events that regulate host responses to materials, occur at the biomaterial–tissue interface, and the physicochemical properties of the material surface modulate these biological events [2,3]. For instance, the chemical properties of the surface of a vascular substitute control blood compatibility (i.e., protein adsorption, platelet adhesion, thrombogenicity, patency). Hence, modification of biomaterial surfaces represents a promising route to engineer biofunctionality at the material–tissue interface to modulate biological responses without altering material bulk properties.

Overview of Surface Modification Strategies

Numerous surface modification approaches have been developed for all classes of materials to modulate biological responses and improve device performance. Applications include the reduction of protein adsorption and thrombogenicity, control of cell adhesion, growth and differentiation, modulation of fibrous encapsulation and osseointegration, improved wear and/or corrosion resistance, and potentiation of electrical conductivity [1]. Surface modifications fall into two general categories: (1) physicochemical modifications involving alterations to the atoms, compounds, or molecules or topography on the surface; and (2) surface coatings consisting of a different material from the underlying support. Physicochemical modifications include chemical reactions (e.g., oxidation, reduction, silanization, acetylation), etching, and mechanical roughening or polishing and patterning (Fig. 37.1). Overcoating alterations are composed of grafting (including tethering of biomolecules), noncovalent and covalent coatings, and thin-film deposition (Fig. 37.2).

Whereas the specific requirements of the surface modification approach vary with application, several characteristics are generally desirable. Thin surface modifications are preferred for most applications because thicker coatings often negatively influence the mechanical and functional properties of the material. Ideally, the surface modification should be confined to the outermost molecular layer (~10–15 Å), but in practice, thicker layers (10–100 nm) are used to ensure uniformity, durability, and functionality. Stability of the modified surface

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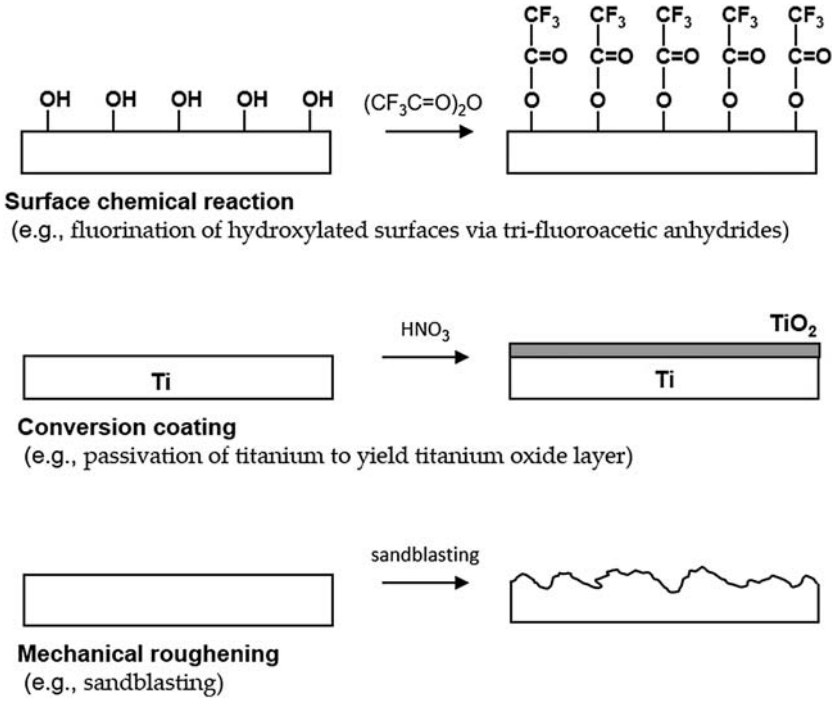

FIGURE 37.1 Schematic representations of common physicochemical surface modifications of biomaterials.

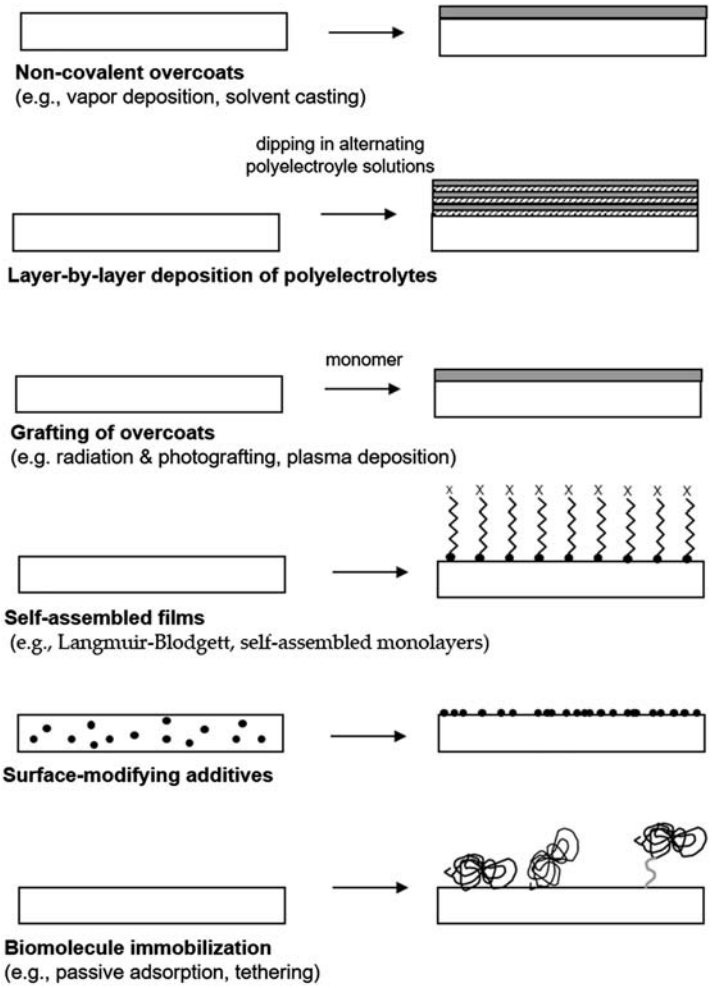

FIGURE 37.2 Schematic representations of common overcoating technologies for surface modification.

is a critical requirement for adequate biological performance. Surface stability refers to mechanical durability (i.e., resistance to cracking, delamination, and debonding) but also to chemical stability, especially in aggressive, chemically active environments such as the biological milieu. Several types of surface rearrangements, such as translation of surface atoms or molecules in response to environmental factors and mobility of bulk molecules to the surface, and vice versa, readily occur in polymers and ceramics after exposure to biological fluids.

Given the uniquely reactive nature and mobility or rearrangement of surfaces, as well as the tendency of surfaces to contaminate readily, rigorous analyses of surface treatments are essential to surface modification strategies. Surface analyses technologies generally focus on characterizing topography, chemistry or composition, and surface energy [4] (Table 37.1). Important considerations for these surface analyses technologies include operational principles (impact of high-energy particles or X-rays under ultrahigh vacuum, adsorption, or emission spectroscopies), depth of analysis, sensitivity, and resolution. For most applications, several analyses techniques must be used to obtain a complete description of the surface.

PHYSICOCHEMICAL SURFACE MODIFICATIONS

Physicochemical modifications involve alterations to the atoms, compounds, or molecules and/or topography on the material surface (Fig. 37.1).

Chemical Modifications

Countless chemical reactions, including UV and laser irradiation and etching reactions to clean, alter, or cross-link surface groups, have been developed to modify biomaterial surfaces [1]. Nonspecific reactions yield a distribution of chemically distinct groups at the surface; the resulting surface is complex and difficult to characterize owing to the presence of different chemical species in various concentrations. Nevertheless, nonspecific chemical reactions are widely used in biomaterials processing. Examples of nonspecific reactions include radio-frequency glow discharge in different plasmas (e.g., oxygen, nitrogen, argon), corona discharge in air, oxidation of metals, and acid–base treatments of polymers. In contrast, specific chemical reactions target particular chemical moieties on the surface to convert them into another functional group with minimal side (unwanted) reactions. Acetylation, fluorination of hydroxylated surfaces via trifluoroacetic anhydrides, silanization of hydroxylated surfaces, and incorporation of glycidyl groups into polysiloxanes are examples of specific chemical reactions. In addition, various chemical methods exist to tether biomacromolecules onto available anchoring groups on surfaces, as described in the Biological Modification of Surfaces section.

The reaction of metal surfaces to produce an oxide-rich layer that conveys corrosion resistance, passivation, and improved wear and adhesive properties (also referred to as conversion coatings) is a common surface modification in metallic biomaterials. For example, nitric acid treatment of titanium and titanium alloys to generate titanium oxide layers is regularly performed on titanium-based medical devices, and the excellent biocompatibility properties of titanium are attributed to this oxide layer [5]. Implantation of ions into surfaces by beaming accelerated ions has been applied to modify the surface properties of metals and ceramics. For example, ion beam implantation of nitrogen into titanium and boron and carbon into stainless steel improves wear resistance and fatigue life, respectively [6]. In addition, evidence suggests that ion beam implantation of silicone and silver can enhance the blood compatibility and infection resistance of silicone rubber catheters [7,8].

Topographical Modifications

The size and shape of topographical features on a surface influence cellular and host responses to the material. For example, surface macrotexture and microtexture alter cell adhesion, spreading, and alignment [9,10] and can regulate cell phenotypic activities, including neurite extension and osteoblastic differentiation [11,12]. Moreover, surface topography can have significant in vivo effects. For instance, the implant porosity modulates bone and soft tissue ingrowth [13,14], and the surface texture alters epithelial downgrowth responses to percutaneous devices and inflammatory reactions and fibrous encapsulation to materials implanted subcutaneously [15–17]. Although specific surface texture parameters that elicit particular biological responses have been identified in several cases, the mechanisms generating these behaviors remain poorly understood.

Methods for generating surface texture can be grouped into approaches for engineering either roughness or topography (Fig. 37.3). Surface *roughness* indicates a random or complex pattern of features of varying amplitude and spacing, typically on a scale smaller than a cell (10–20 μm). On the other hand, surface *topography* refers to

TABLE 37.1 Common Surface Analysis Techniques

	Principle	Operation	Spatial Resolution	Depth	Sensitivity	Texture	Chemical Composition			
							Elements	Compounds	Isotopes	Additional
Contact angle	Liquid wetting of surfaces	Air Liquid	NA	3–20 Å	NA	Indirect				Surface energy
Atomic force microscopy	Records interatomic forces between tip and sample	Air Aqueous	Atomic	NA	Single atom	Yes	No	No	No	
Scanning electron microscopy	Secondary electron emission caused by electron bombardment is imaged	Vacuum	40 Å	5–10 Å	High	Yes	No	No	No	Crystallinity
Energy-dispersive X-ray spectroscopy	X-ray emission caused by electron bombardment	Vacuum	40 Å	1 μm	10^{-7} g/cm^2	No	$Z > 5$	No	No	
Auger electron spectroscopy	Auger electron emission caused by electron bombardment	Vacuum	100 Å	15–50 Å	10^{-10} g/cm^2 0.1 atm%	No	$Z > 3$	Chemical shift	No	
X-ray photoelectron spectroscopy	X-rays cause emission of photoelectrons with characteristic energies	Vacuum	10 μm	10–150 Å	10^{-10} g/cm^2 0.1 atm%	No	$Z > 3$	Chemical shift (excellent)	No	
Secondary-ion mass spectrometry	Ion bombardment causes secondary ion emission	Vacuum	3-10 μm	10 Å	10^{-13} g/cm^2	No	All	Yes	Yes	
Fourier transform infrared (IR) resonance	Molecular vibrations resulting from adsorption of IR radiation	Air Aqueous (attenuated total reflection)	10 μm	<1 μm	1 mol%	No	Indirect	Vibration frequency	No	Monolayer orientation

NA, not available.

FIGURE 37.3 Surface roughness and topography.

patterns of well-defined, controlled features on the surface. Surface roughness has been traditionally modified via sandblasting, plasma spraying, chemical etching, and mechanical polishing; the nonspecific nature of these processes renders surfaces with random or complex topographies. Ion beam and electric arc (for conductive materials) texturing approaches have also been applied to modulate surface roughness. To generate controlled topographies, micromachining and nanomachining techniques have been exploited using silicon, glass, and polymers as substrate materials [10]. Photolithography combined with reactive plasma and ion etching has been extensively applied to generate surfaces with well-defined topographies. This technique enables the preparation of machined silicon and polymeric substrates and silicon templates that can then be used as molds to transfer features to polymers via solvent casting or injection molding. Similarly, *Lithographie, Galvanoformung, Abformung* electron beam and laser machining have been used to manufacture defined topographical features on various materials. Alternatively, hot-embossing imprint lithography represents a robust approach to the low-cost and rapid fabrication of microscale and nanoscale features on biomedically relevant polymers [18].

OVERCOATING TECHNOLOGIES

Coating strategies rely on the deposition of a surface layer consisting of a different composition from the underlying base material (Fig. 37.2). These surface modification approaches include noncovalent and covalent coatings [1].

Noncovalent Coatings

Major advantages of noncovalent coatings include simple application and the ability to coat a variety of different base materials. Examples of common noncovalent coating methods are solvent casting and vapor deposition of metals, parylene, and carbons. In the Langmuir–Blodgett deposition method, one or more highly ordered layers of surfactant molecules (e.g., phospholipids, amphiphiles) are placed at the surface of the base material via assembly at the air–water interface and compression of the surfactant molecules. Langmuir–Blodgett films exhibit high order and uniformity and provide flexibility in incorporating a wide range of chemistries. The stability of these films can be improved by cross-linking or internally polymerizing the surfactant molecules after film formation. Polymer coatings containing drugs can be applied by solvent dip-coating or spraying over the substrate. This is widely used to make drug eluting stents (Cypher, Cordis Corporation; and TAXUS, Boston Scientific Corporation) that result in a lower risk for restenosis [19]. However, coatings should be thin and mechanically stable because defects in such polymer coatings have potential risks for thrombosis and coronary microembolism [20]. Another surface modification strategy that takes advantage of intermolecular interactions is the deposition of multilayer polyelectrolytes (e.g., poly[styrene sulfonate]–poly[allylamine], hyaluronic acid–chitosan). In this simple layer-by-layer (LbL) method, a charged surface is sequentially dipped into alternating aqueous solutions of polyelectrolytes of opposite charge to deposit thin multilayers of a polyelectrolyte complex [21]. This technique enables the deposition of films on any topography and material in an aqueous environment. For instance, alternating layers of chitosan (positively charged) and hyaluronic acid (negatively charged) were deposited on titanium implants using LbL self-assembly and resulted in reduced bacterial adhesion because of the antibacterial properties of chitosan [22]. Another elegant strategy for surface modification is to use surface-modifying additives. These molecules are blended in the bulk material during fabrication but will spontaneously rise to and concentrate at the surface owing to the driving force to minimize interfacial energy; they have been shown to increase the hemocompatibility and stability of polymers such as polyurethane and poly(lactic-*co*-glycolic acid) [23]. Mussel-inspired surface modification strategies have gained significant interest. Mussels attach to all kinds of organic and inorganic surfaces using catechol groups on their adhesive proteins in aqueous environments [24]. Polymers containing catechol groups can be deposited by dip-coating in an aqueous solution to form the desired outer surface coatings over the base material.

Covalent Coatings

Covalent coating methodologies rely on direct tethering of overcoats onto the base material to improve film stability and adherence. Radiation grafting, with ionizing radiation and high-energy electron beams, and photografting have been extensively pursued to modify polymer substrates to introduce chemically reactable groups into inert hydrophobic polymers and polymerize overcoats onto the base support [1]. In principle, the radiation breaks chemical bonds in the base material into free radicals and other reactive species, which are then exposed to a monomer or presynthesized polymers. Use of presynthesized polymers is referred to as "grafting-to" approaches, and although it provides control over the molecular weight of the overcoat, the density of grafts is limited by the steric crowding of polymer chains. "Grafting-from" approaches involve a monomer reaction with the reactive species at the surface and propagation as a free radical chain reaction. Because of the use of low−molecular weight monomer, dense polymer brushes can be synthesized. These strategies enable the generation of a wide range of surface chemistries; unique graft copolymers can be synthesized by combining different monomers. Using such approaches, protein-resistant brush polymers can be produced on a variety of surfaces and can reduce bacterial colonization significantly. Plasma deposition (also referred to as glow discharge deposition) via radio-frequency or microwave has also been extensively applied to biomaterial surface modification [25]. In particular, radio-frequency glow discharge plasma deposition has received considerable attention because it can generate continuous conformal coatings (relatively free of pinholes and voids) that can be applied to many different types of supports (metals, ceramics, and polymers) with complex geometries. In addition, these films exhibit good adherence to the substrate and can be engineered to present different functionalities, although the resulting chemistry is complex and ill-defined. In contrast to these relatively low-energy/low-temperature plasmas, high-energy/high-temperature plasmas have been used to apply inorganic surface modifications onto inorganic substrates. For example, calcium phosphate ceramic particles such as hydroxyapatite have been deposited via flame-spraying onto titanium and cobalt chrome orthopedic implants to improve osseointegration [26].

Coatings consisting of self-assembled monolayers (SAMs) have gained significant attention as robust surface modification agents [27,28]. These films spontaneously assemble, form highly ordered, well-defined surfaces with excellent chemical stability, and provide a wide range of available surface functionalities. The basic structure of molecules that form SAMs is an anchoring "head" group, organic chain backbone, and functional "tail" group. Common SAM systems are alkanethiols on coinage metals (gold and silver), n-alkyl silanes on hydroxylated supports (glass and silica), and phosphoric acid or phosphate groups on titanium or tantalum surfaces. Assembly of these organic chains into highly ordered structures is driven by the strong adsorption of the anchoring "head" group of the monolayer constituent to the surface and van der Waals interactions of the backbone chains. The order and stability of the SAMs are strongly influenced by the length of the backbone chain; in the case of alkanethiols, molecules with backbones with 9−24 methylene groups assemble well on gold. Importantly, the terminal functional group is presented at the surface−solution interface and controls the physicochemical properties of the SAM.

BIOLOGICAL MODIFICATION OF SURFACES

Biomolecules (e.g., cell receptor ligands, enzymes, antibodies, pharmacological agents, lipids, nucleic acids) have been immobilized onto and within biomaterial supports for numerous therapeutic, diagnostic, and bioprocess applications. Table 37.2 lists examples of biological modifications to surfaces for biomedical and biotechnological applications. The rationale for these hybrid materials integrating synthetic and biological components is to convey biofunctionality and hence engineer materials that elicit desired biological responses or have attributes associated with biosystems. One of the earliest examples of this strategy is the immobilization of heparin onto polymer surfaces to improve blood compatibility. Drug-eluting stents (stents coated with a polymeric layer loaded with antihyperplasia drugs) have also been developed to reduce restenosis and improve patency. Another example of a widely used biological modification strategy is the immobilization of adhesive ligands, either adsorbed proteins (e.g., fibronectin, laminin) or tethered synthetic oligopeptides (e.g., arginine-glycine-aspartate [RGD]), on synthetic and natural supports to promote cell adhesion and function in various tissue engineering and regenerative medicine applications [29]. Important considerations in modifying surfaces with biological molecules include the density, distribution (uniform versus clustered), and activity (e.g., orientation, conformation, accessibility) of the immobilized biomolecule.

TABLE 37.2 Biomedical and Biotechnological Applications of Immobilized Biomolecules

Biomolecule	Applications
Heparin	Blood-compatible surfaces; growth factor immobilization
Fibronectin, collagen Arginine-glycine-aspartate peptides	Cell adhesion and function in biosensors, arrays, devices, and tissue-engineered constructs
Antibodies	Biosensors; bioseparations; anticancer treatments
DNA plasmids Antisense oligonucleotides Small interfering RNA	Gene therapy for a multitude of diseases; DNA probes
Growth factor proteins and peptides	Anticancer treatments; treatments for autoimmune and inflammatory conditions; enhanced wound repair
Enzymes	Biosensors; bioreactors; anticancer treatments; antithrombotic surfaces
Drugs and antibiotics	Antithrombotic agents; anticancer treatments; antihyperplasia treatments; antiinfection/inflammation treatments
Polysaccharides	Nonfouling supports for biosensors and bioseparations

Three major methods are used to immobilize biomolecules onto biomaterial surfaces: physical adsorption, physical entrapment, and covalent immobilization (Fig. 37.4) [30]. Passive physisorption of biomacromolecules (i.e., proteins, polysaccharides, nucleic acids) is a simple yet efficient method to render surfaces biologically active. Everyday applications include the coating of synthetic materials with extracellular matrix proteins such as fibronectin and collagen to improve cell adhesion. Protein adsorption is a complex, dynamic, energy-driven process involving hydrophobic interactions, electrostatic interactions, hydrogen bonding, and van der Waals forces. Protein parameters such as the primary structure, size, and structural stability, as well as surface properties including surface energy, and the chemistry influence the biological activity of the adsorbed biomacromolecules. These biologically modified surfaces can undergo further modifications, such as the displacement of adsorbed proteins and the cell-mediated deposition and remodeling of matrix components in the biological milieu. As an approach to improving the stability of these modified surfaces, the biological molecules can be cross-linked after adsorption. Finally, the use of high-affinity interactions, such as avidin–biotin and antibody–antigen, represents a special case of these physical immobilization methods that is particularly important in diagnostics and bioprocessing.

Physical entrapment methods rely on diffusive barriers or matrix systems to control the transport or availability of the biomolecule. For example, entrapment of enzymes within sol-gels with nanoscale porosity and drug or protein therapeutics within encapsulation matrices provide technologies for enhanced stability, separation, or recovery of the biological agent, and regulated delivery kinetics. The encapsulation systems can be engineered to isolate the biomolecule permanently or degrade in nonspecific (e.g., hydrolysis) or specific (e.g., enzymatic degradation) fashion for controlled-release kinetics.

An extensive and diverse group of strategies has been developed to immobilize or tether biomolecules covalently to soluble or solid supports (Fig. 37.4) [30,31]. Soluble polymers functionalized with biomolecules can then be polymerized into a network or grafted onto a solid support. These strategies rely on coupling reactions between groups in the biomolecule ($-NH_2$, $-COOH$, and $-SH$) and the biomaterial support, and often involve cross-linkers or coupling agents such as CNBr, carbodiimides, and *N*-hydroxysulfosuccinimide. Furthermore, chemoselective reactions such as "click" chemistry, provide unparalleled control over the tethering and presentation of biomolecules that are bio-orthogonal and hence provide specificity without unwanted side reactions [32]. In many instances, the biomolecule is covalently immobilized via an inert spacer arm (e.g., polyethylene glycol) that provides increased steric freedom and activity. In addition, the tether arm can be designed to be hydrolytically or enzymatically labile to allow for release of the tethered biomolecule. As expected, the properties of the underlying biomaterial support have central roles in the tethering efficiency and resulting biological activity of the immobilized biomolecule. In some cases, the surface needs to be modified via the techniques described earlier to introduce reactive groups for the subsequent immobilization step. For example, inert surfaces can be modified by overcoating with a polymeric adlayer that then presents anchoring groups suitable for the immobilization of biomolecules. For many

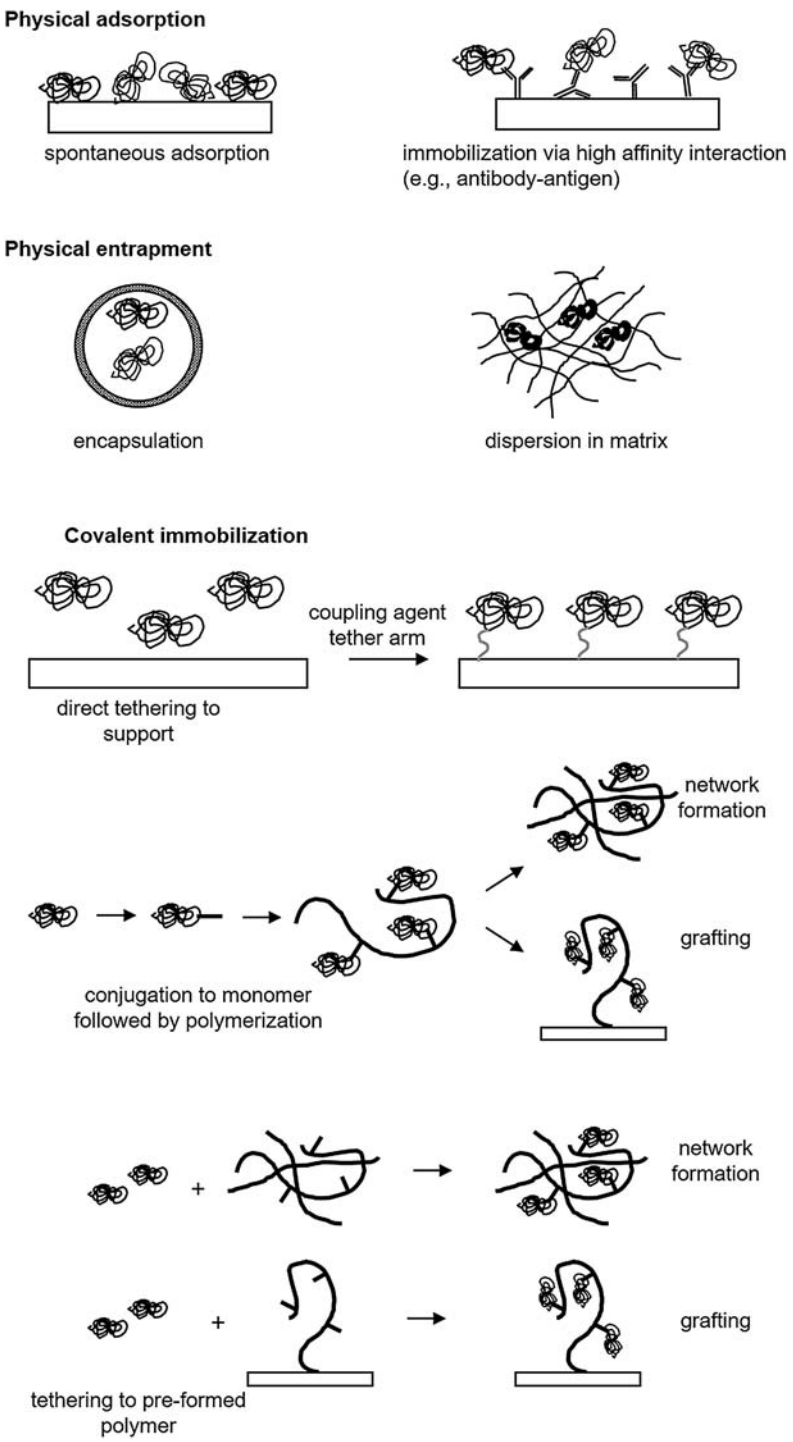

FIGURE 37.4 Schematic diagram of methods for immobilizing biomolecules onto and within biomaterials.

biomedical and biotechnological applications, it is desirable to tether biomolecules within a protein adsorption-resistant (nonfouling) background to eliminate effects associated with nonspecific protein adsorption. This is particularly important in biomaterials and regenerative medicine applications in which inflammatory responses to nonspecifically adsorbed proteins limit biological performance. Poly(ethylene glycol) (PEG) ($-[CH_2CH_2O]_n$) groups have proven to be the most protein-resistant functionality and remain the standard [33]. A strong correlation exists between PEG chain density and length and resistance to protein adsorption, and consequently cell adhesion. Other hydrophilic polymers such as poly(2-hydroxyethyl methacrylate), polyacrylamide, polycarboxybetaine, and

phosphoryl choline polymers also resist protein adsorption. In addition, mannitol, oligomaltose, and taurine groups have emerged as promising moieties to prevent protein adsorption [34,35]. Critical considerations for these approaches include the ability to apply these coatings to existing medical-grade materials and the long-term stability of these coatings in terms of their nonfouling character and whether protein adsorption can be reduced below threshold levels to suppress nonspecific biological responses. Emerging strategies combining passive and active characteristics (e.g., biotherapeutics that modulate blood clotting or inflammation, self-cleaning materials) provide promising approaches to reduce protein adsorption effectively while enabling the controlled presentation of bioactive molecules.

SURFACE CHEMICAL PATTERNING

Whereas the surface chemical and biological modification strategies described here were presented in the context of a uniform surface, many of these technologies can be used to generate surfaces that have chemical or biological functionalities in distinct geometrical patterns. Important applications of patterned surfaces include protein and oligonucleotide arrays, biosensors, and cell-based arrays [36]. In many instances, these patterned substrates contain spatially defined domains with biomolecules surrounded by a nonfouling background. Photolithography and other techniques relying on exposure through masked patterns or direct surface exposure (e.g., laser or electron beam) combined with chemical reaction or grafting are often used to generate chemically patterned surfaces. Importantly, "soft" lithography methods such as microcontact printing and microfluidic fluid exposure have been applied to produce micropatterned substrates in high throughput at a low cost and without the need for a cleanroom environment [37].

CONCLUSION AND FUTURE PROSPECTS

Surface modifications of biomaterials represent promising routes to engineer biofunctionality at the material–tissue interface to modulate biological responses without altering material bulk properties. Countless technologies have been developed to create physicochemical modifications involving alterations to the chemical groups on the surface and coatings consisting of a different material from the underlying support, including immobilized biomolecules. These approaches hold tremendous promise to enhance biomaterial performance in regenerative medicine. Future structure–function analyses on the effects of specific surface chemistries, topographies, and biological modifications on in vivo responses, especially in healing and regenerative environments, will further advance the understanding of host responses to implanted devices. These insights will result in the identification of surface modifications that synergize with biological elements (e.g., cells, growth and differentiation factors) to enhance tissue repair and regeneration. It is anticipated that technical breakthroughs in synthetic chemistry, biofunctionalization, microfabrication and nanofabrication, and surface characterization will lead to the engineering of advanced bioactive materials. In particular, complex patterns of bioligand presentation, such as clusters, gradients, temporal exposure, and multiple ligands, are expected to provide unparalleled control over cellular activities and healing responses.

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CHAPTER

38

Histogenesis in Three-Dimensional Scaffolds

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THE NEED FOR REPLACEMENT TISSUES

Replacement of diseased tissues and organs is one of the biggest problems facing the medical industry. According to the US Scientific Registry of Transplant Recipients, there were over 97,000 patients on the National Organ Registry at the end of 2007 [1]. Of the patients awaiting life-saving transplants, approximately 10% died before they could receive donor organs. In addition, tissue disease and organ failure lead to an estimated 8 million surgical procedures annually in the United States [2]. From these statistics, one fact is clear: the need for replacement organs far outweighs the supply, and the discrepancy is only expected to worsen as populations rise and life expectancies increase.

Synthetic materials and tissue grafting have been employed throughout history in an effort to address the need for tissue substitutes, although each is marred by seemingly insurmountable challenges. In the case of synthetic replacements, the primary mode of failure comes through attack via the host's foreign body response. Disease transmission, in the case of xenografts or allografts, and donor site morbidity from autografts leave these tissues underused, although the primary constraint, as with whole organs, is a limited supply. Thus, tissue engineering, with its goal of recapitulating biological structure and function, has emerged as the most viable solution to the lack of suitable replacement tissues.

The idea that tissue function can be restored is as old as the medical profession. Historically, one of the most popular sites of implant was the nose, which, other than the hand, was the body part most likely to be injured in battle [3]. As a consequence, many cultures were performing successful nasal operations thousands of years ago. A pioneering technique for nasal tissue regeneration was developed in 1596, when a flap of skin from the arm, complete with underlying vessels, was reconnected to the face. In many cases, these tissues were able to reconnect with the local blood supply and successfully regenerate [4]. By the 19th century, numerous surgeons had successfully transplanted skin between individuals [4,5] and with World War II came the advent of large-scale tissue replacement using synthetic materials. Faced with high numbers of battlefield casualties, surgeons began experimenting with artificial materials for tissue replacement and augmentation in efforts to reduce battlefield mortality [6]. Although most of these attempts were unsuccessful for various reasons, the idea of replacing lost tissue with synthetic materials endured and continues to be used today.

Through the successes and challenges of previous replacement strategies, the medical community has gained a heightened appreciation for the complexity of biological tissues. It is recognized that each tissue is a carefully organized aggregate of cells and other factors specialized to perform a particular function [7], and that the complexity increases as tissues come together to form organs. Although still relying to a large degree on artificial materials and devices, the focus of tissue replacement research is moving toward regenerative medicine and total biological solutions. Along those lines, this chapter will focus on efforts to promote histogenesis in three-dimensional (3D) scaffold matrices.

TISSUE COMPONENTS

Biological tissues are composed of three components: cells, the extracellular matrix (ECM), and the signaling systems that are encoded by genes in the nuclei of the cells and then activated through cues from the ECM or other cells

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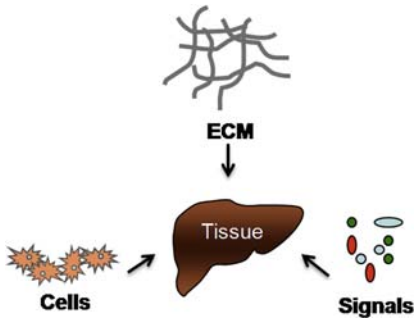

FIGURE 38.1 The basic components of mammalian tissue. *ECM*, extracellular matrix.

[8] (Fig. 38.1). Together, the three components interact in balance to form tissues and organs; mimicking these interactions is the focus of tissue engineering.

From an engineering design standpoint, the tissues of the human body are infinitely complex, and the dynamic processes that constitute organs are not confined to a single dimension. At the most basic level, cells reside in a complex ECM environment of proteins, carbohydrates, and other structural molecules. Cells respond to changes in their environment (e.g., injury or disease) by secreting proteases, growth factors, and other signaling molecules, which in turn lead to intramolecular and intercellular communications dictating the initiation of processes from proliferation and protein synthesis to migration and apoptosis.

The ECM can be broadly defined as a natural scaffold that supports tissues and organs, but it should not be viewed as merely providing strength and physical reinforcement. This matrix, which is composed of both a fibrillar and an amorphous component, is known to be intricately involved in the events that lead to tissue formation [9]. These two broad components interact with cells via cell surface receptors and various membrane proteins to influence processes such as cell orientation, growth and differentiation, and the secretion of bioactive molecules [9]. Cellular responses to changes in the tissue environment may result from direct interaction with the matrix, such as when physical stresses are transmitted through fibrillar ECM components to cell focal adhesion complexes within the cell, or they may arise as a consequence of the release of signaling molecules that are stored in the ECM reservoir. Understanding the complexity of tissue components and their roles in natural processes, such as embryogenesis and wound healing, will guide tissue engineering methodologies and lead to more functional replacement options.

REGENERATION OF DISEASED TISSUES

Regeneration is defined as the synthesis of physiologic tissue with the objective of restoring lost biological function. This is in contrast to the notion of tissue repair, which is simply the closure of a wound to allow for the return of homeostatic function at the site of injury [10]. Repair generally results in the contraction of tissue and the formation of a scar, which contains undifferentiated epithelial tissue with little or no physiological function. Although many tissue engineering strategies have been influenced by the principles at work in the wound healing process (especially with regard to cell−cell and cell−matrix interactions), the formation of scar tissue is regarded as an adverse event that directly inhibits the regeneration of new tissues [11].

Unlike an embryo, the adult human has limited capacity for self-regeneration. The precise reason for this inability is not understood, but research demonstrates that the removal of ECM, as in disease or injury, disrupts normal tissue regeneration and leads directly to the formation of scar tissue [10]. This observation signifies the importance of a structural support to direct and guide cell-mediated regeneration. Indeed, studies have shown that for large tissue defects, the implantation of cells alone is ineffective at initiating new tissue formation [11]. Depending on the size and location of tissue damage, cells must work in conjunction with the ECM to initiate repair or regeneration. Again, it is valuable to consider the natural process of wound healing when attempting to delineate these interactions.

Tissues are composed of three interconnected layers: the epithelium, basal lamina, and stroma. The epithelium is composed entirely of cells with little or no matrix component. In contrast, the basal lamina contains only ECM, whereas the stroma is composed of cells, ECM, and blood vessels [12] (Fig. 38.2). Both the epithelia and basal lamina can regenerate without inducing scar formation, but the stroma is usually nonregenerative, and injury to this layer leads directly to the repair state [13]. When tissue injury occurs, the inflammatory response is swift and quickly

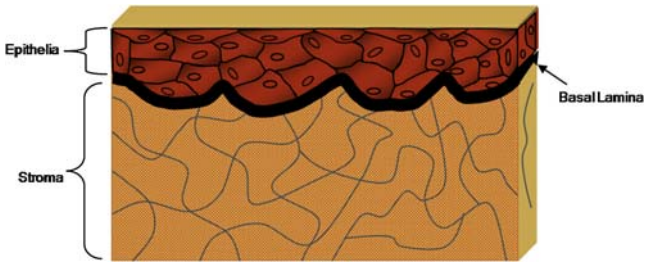

FIGURE 38.2 The three layers of tissue. The epithelia is the outermost layer of tissue and is composed mainly of cells. The basal lamina is a thin extracellular matrix (ECM) membrane that separates the epithelia from the stroma. The stroma is mainly composed of ECM fibers and is generally unable to regenerate spontaneously.

results in recruitment of fibroblasts and myofibroblasts to the wound bed [13]. The destruction of stromal tissue signals myofibroblasts to secrete collagen fibrils in an attempt to stabilize the wound. Unlike the random fibril orientation achieved by fibroblasts in normal tissue formation, myofibroblasts deposit collagen in a highly organized fashion along stress planes [10]. The stress forces generated by these contractile cells are generally in one dimension and both the myofibroblasts and matrix fibers align parallel to the force [14]. Deposition of ECM under stress results in contraction of the wound bed, bringing healthy tissue closer together and thus reducing the size of the defect to be healed. Unfortunately, the highly aligned scar fibrils inhibit subsequent infiltration by additional cell types, thereby limiting the extent to which the scar can be remodeled into functional, regenerated tissue.

With the goal of limiting wound contraction and subsequent scar tissue formation, much research has focused on implanting exogenous materials into the defect site to replace the lost matrix component. As example, Yannas and colleagues placed a collagen scaffold into a full-thickness skin wound and were able to block wound contraction and greatly reduce scar formation while inducing partial skin regeneration [13]. The collagen construct was shown to reduce the inflammatory response greatly, and consequently, the number of myofibroblasts recruited to the wound bed. In control studies, inhibition of contraction and subsequent tissue regeneration was not evident in defects treated with cytokines or cell suspensions, or in artificial scaffolds known to induce a high inflammatory response [11]. In addition, inhibition of contraction by steroids or other chemicals [15] is not sufficient for regeneration, which demonstrates the complex influence of scaffold materials in the healing process.

Although the need for a scaffold material has been validated, a complete tissue replacement strategy is composed of more than a single component. Successful histogenesis requires the presence of cells capable of producing new tissue matrix and must include the biochemical factors necessary for the cells to live and function. Furthermore, several important design parameters related to the scaffold itself have become evident. In addition to being biocompatible, scaffold materials should have the appropriate chemical structure and microarchitecture to support cell viability and tissue formation. As tissues mature, precisely tuned scaffold degradation will allow the transfer of mechanical properties to the newly formed matrix and materials permissive to vascular ingrowth will encourage incorporation with surrounding tissues and provide a blood supply for nourishment.

DESIGN PARAMETERS FOR HISTOGENESIS

Tissue engineers have recognized certain critical components to regeneration design strategies. Methodologies generally start by including the three components of tissue: cells, matrix, and signaling factors. From here, constituents are specialized based on specific tissue needs. In general, researchers seek to develop a scaffold material that can support viability of the appropriate cell type while acting as a temporary substitute for the ECM. Over time, this surrogate matrix will ideally be replaced by functional replacement tissue.

Cell Sources

Because scaffold materials alone are insufficient to stimulate regeneration of all tissue defects, cell sourcing is an important consideration in the histogenesis design strategy. Host cells have been shown to infiltrate acellular matrices in some instances, but generally this infiltration distance is limited to a few microns. By implanting cells into the biomaterial scaffold, it is believed that the time for tissue infiltration by the host will be minimized and

the cells can secrete bioactive factors in vitro that will encourage autologous ECM formation. Thus, a combination of cells and scaffold serves as the most common implant construct.

A variety of cell types have been investigated for regeneration applications, including differentiated cells, adult-derived stem cells, and embryonic stem cells [16]. Mature cells have historically come from three sources, autologous, allogeneic, and xenogeneic, although xenogeneic cell transplantations have mostly been abandoned because of concerns regarding immune rejection and cross-species disease transmission. Allogeneic cells are harvested from healthy adult donor organs and then expanded in vitro. Scaffolds with allogeneic cells are subject to immune rejection, but these cells have been successful in skin regeneration for burn patients [17]. Autologous cells biopsied from a patient, expanded in vitro, and then seeded onto a tissue scaffold are generally viewed as the ideal replacement in terms of compatibility. However, in many patients, the extent of tissue damage or disease makes it difficult to obtain a sufficient supply of healthy cells [16].

The field has seen an increase in the number of progenitor cells employed for regeneration applications. These stem cells have varying degrees of potential, depending on their source, but in general, adult-derived cells are considered multipotent and have the capacity to generate a limited number of cell types, whereas pluripotent, embryonic cells can be differentiated into cells of all three germ layers [18]. As example, murine embryonic stem cells have been differentiated in vitro into various tissue cells including neurons, myocytes, and chondrocytes [19]. This differentiation was initiated in vitro through the use of media containing cytokines and growth factors specific to the target cell lineage. Osteoblast differentiation has been accomplished in a similar manner and by incubating embryonic stem cells in preconditioned media transferred from a culture of mature bone cells [20]. Mechanical stimulation can also serve as a tool to guide differentiation for orthopedic [21] or cardiovascular [22] applications of embryonic stem cells. The advent of induced pluripotent stem cells, derived from reprogramming adult cells such as fibroblasts, further expands possibilities for the field [23].

Porosity

In most types of synthetic materials, successful histogenesis requires a porous microstructure. Scaffold porosity, pore size, and the overall pore structure all have important effects on tissue formation and infiltration into biomaterial constructs. Interconnecting pores facilitate the loading of cells into scaffold materials whereas the increased internal surface area provides sites for attachment and spreading. By allowing the diffusion of metabolites, oxygen, and growth factors into and out of the material, an open network structure permits cell viability and proliferation and allows room for the deposition of cell-secreted proteins. In vivo, a porous structure serves to encourage the infiltration of host cells and, importantly, the extension of vascular processes that offer nourishment to developing tissue.

Methods of creating pores or increasing the porosity of biomaterials include gas foaming [24], salt leaching [25], freeze-drying [26], and electrospinning [27], and the inclusion of biodegradable hydrogel microparticles [28]. As example, a microporous polyurethane urea was developed using a gas-foaming method. In these studies, sodium bicarbonate is added to a mixture of a polyurethane urea copolymer in dimethylformamide. The polymer is cast in a mold and the solvent evaporated. The cast polymer is next placed into an acidic solution to react with the salt. Both the removal of the salt and the bubbles formed during this foaming process contribute to the porosity of the resulting scaffold [29]. The final material is homogeneous in structure with a surface pore morphology and pore volume fraction similar to that seen throughout the bulk (Fig. 38.3). In addition, incorporation of the

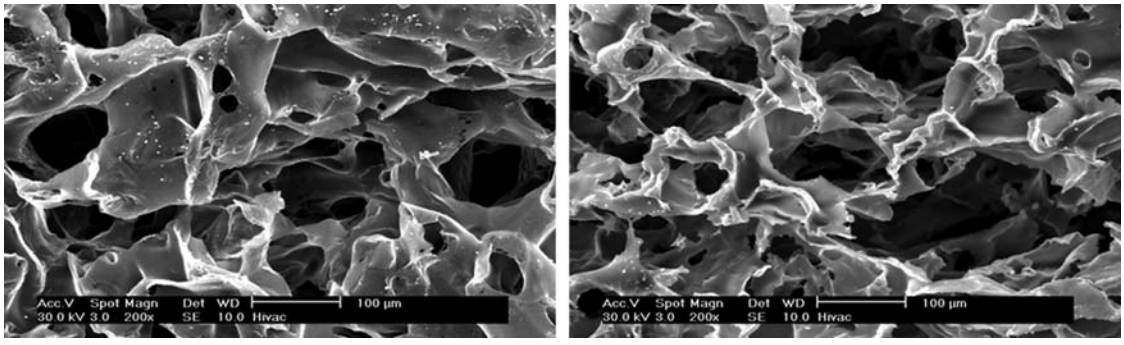

FIGURE 38.3 **Microporous polyurethane urea copolymer scaffold.** The scaffold formed by gas foaming exhibits a similar porous morphology on the surface (*left*) and in the bulk (*right*).

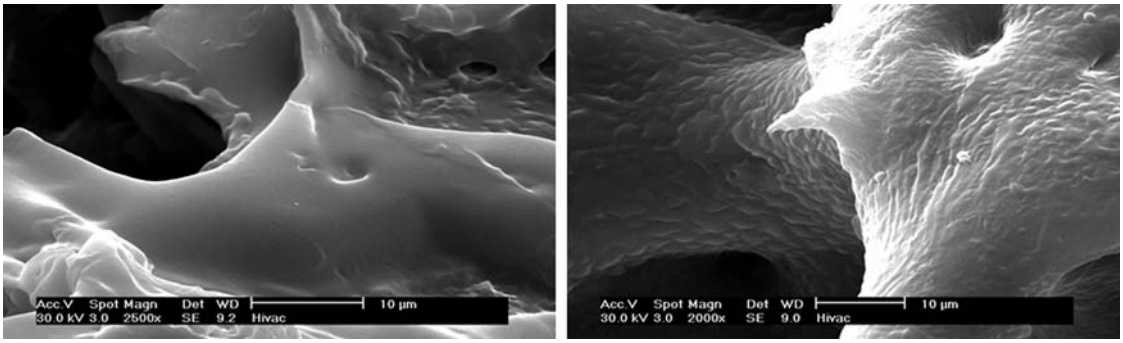

FIGURE 38.4 **Microporous polyurethane urea scaffold modified for cell adhesion.** The original scaffold porosity (*left*) was unaltered by the addition of the cell-adhesive peptide YIGSR (*right*).

laminin-derived peptide YIGSR permits cell adhesion to the otherwise nonadhesive polymer matrix without changing the overall porosity of the scaffold (Fig. 38.4).

It is important to consider how variations in pore architecture affect cell–material interactions. Experimental studies have led to the determination of a characteristic interaction parameter, Φ_c, which relates the number of cells bound, N_c, to the available surface area, A, of the scaffold, according to Eq. (1) [10]:

$$\Phi_c = \frac{N_c}{A}, \tag{38.1}$$

By careful analysis, it can be shown that two matrices with identical chemistries but differing pore diameters have vastly different abilities to provide for cellular attachment. For example, scaffolds containing pores of 300 μm have a much lower internal surface area than do matrices with pore diameters of less than 50 μm, and as such, they bind a much lower number of cells in the matrix. Because of the low cellular infiltration, tissue formation in these constructs is slow and in certain cases inhibited owing to a lack of interactions between cells [10]. From this calculation, it can be assumed that there is a maximum pore diameter that will allow cells sufficient area to attach and divide while maintaining the critical intracellular communication that leads to histogenesis. This parameter will be influenced by scaffold material properties (e.g., hydrophobicity) and limited by the size of the cell in each application. In general, research supports that pores larger than 10 μm (the average cell diameter) will permit cell infiltration [30].

In terms of successful histogenesis, both the pore size and the degree of porosity are tissue and scaffold specific. For example, in vivo osteogenesis occurs in biomaterials of high porosity (>70%) with average pore sizes larger than 300 μm [31]. However, in skin regeneration, successful scaffolds need only to exhibit pore sizes of 20–125 μm [32]. This discrepancy can be explained by the low vascular requirements of the skin and its convenient juxtaposition to an ample supply of atmospheric oxygen. However, there is an upper limit in the porosity and pore size set by constraints associated with mechanical properties. An increase in the void volume results in a reduction in the mechanical strength of the scaffold, which can be detrimental in applications where regenerated tissues must support significant mechanical loads (e.g., long bones, heart valves, and articular cartilage) [13]. The extent to which the porosity of a scaffold can be increased while allowing it to meet tissue mechanical requirements depends on many factors, including the intrinsic makeup of the biomaterial and the processing conditions used in fabrication [31].

As histogenesis progresses and gives way to organogenesis, the impact of the scaffold pore structure on material degradation and tissue vascularization becomes apparent. The size and distribution of pores within a scaffold greatly influence the manner and rate of in vivo degradation [33], which can affect tissue formation and construct mechanical integrity. In materials susceptible to hydrolytic cleavage, for example, the access of water molecules to the interior of a scaffold is limited by porosity. Similar parallels exist for matrices subject to enzymatic degradation, which rely on interaction with cell-secreted molecules for dissolution. A final, important consideration is the influence of pore structure on the establishment of a blood supply in newly developing tissue. In early stages of histogenesis, nutrients, metabolites and other factors essential to cell survival pass freely through scaffold pores. As these pores fill with new tissue, however, a functioning vasculature is necessary. New "designer" tissue-engineered scaffolds are composed of precisely controlled porous architectures that support and guide vessel ingrowth during tissue development [34,35]. The concepts of degradation and microvascularization are discussed in more detail in the following sections.

Degradation

Although nondegradable biomaterials have had success in many medical devices, complication primarily caused by chronic foreign body responses have yet to be overcome. For this reason, the ideal regeneration construct is one that can eventually be replaced by native tissues. Furthermore, the degradation rate of the construct is intrinsic to the success of the implant. This means that the material dissolution should complement tissue synthesis to ensure suitable mechanical stability during the process of histogenesis. The necessary scaffold residence time is tissue specific and must account for the time required for cells to populate the scaffold adequately and deposit a stable ECM. If a biomaterial degrades before sufficient ECM deposition has occurred, cells will lose important physiochemical factors for tissue regeneration and repair is likely to occur, resulting in scar formation. However, if the scaffold residence time is too long, ECM deposition and cell proliferation will be suppressed. An important balance must be established for successful regeneration.

In most currently employed scaffold materials, degradation in the in vivo aqueous environment occurs via hydrolysis of chemical bonds in the base material. Chemical functionalities, molecular weight, and the degree of cross-linking determine the degradation characteristics. For example, higher−molecular weight materials tend to degrade more slowly over time as do materials with a higher hydrophobicity and crystallinity. Using a combination of these factors, predictable degradation profiles can be developed to match expected tissue formation rates. However, the consequences of material dissolution must be considered. As mentioned earlier, scaffolds that undergo bulk erosion can rapidly become unstable owing to the formation of large pores with low mechanical stability [36]. In addition, the degradation products of some scaffolds can be toxic not only to cells of the surrounding tissue, but also to vital organs of the lymphatic system. For example, degradation of the frequently studied polylactic acid (PLA) and polyglycolic acid (PGA) scaffolds results in a marked drop in pH in the local vicinity because of the release of acidic degradation products [36,37]. The decrease in pH can be detrimental to cells and organs; over time, it can lead to an inflammatory response with possible capsule formation and tissue necrosis [38].

As an alternative to hydrolytic degradation, many investigators are developing smart materials that can be dynamically remodeled during histogenesis via cell-mediated processes. These scaffolds are designed to mimic the degradation of natural ECM proteins, which are subject to matrix metalloproteinases (MMPs) and serine proteases that are either secreted or activated by most cell types. Because proteolysis-induced degradation is required for cell migration and invasion, researchers have had success in introducing synthetic hydrogels that are sensitive to cell proteases. Hydrogels containing amino acid sequences that can be degraded by plasmin [39], MMPs [40], or both of these protease families [41−43] exhibit sustained degradation upon cellular infiltration.

In our own laboratory, we have fabricated MMP-degradable hydrogels that become fluorescent when degraded by cell proteases [44]. These = polyethylene glycol (PEG)-based hydrogels are synthesized with MMP-degradable segments in the polymer backbone that are labeled with fluorescent, self-quenching tags. Thus, intact substrates show no fluorescence, but upon degradation by cell proteases, quantifiable fluorescence is emitted. Cells seeded in these fluorogenic substrates are able to cleave the degradable hydrogel matrix, as visualized by a marked increase in fluorescence in the areas immediately around the cell (Fig. 38.5). In addition, cell migration trails could be seen in the hydrogels. It is believed these materials will contribute to an understanding of cell migration and cell-mediated scaffold degradation.

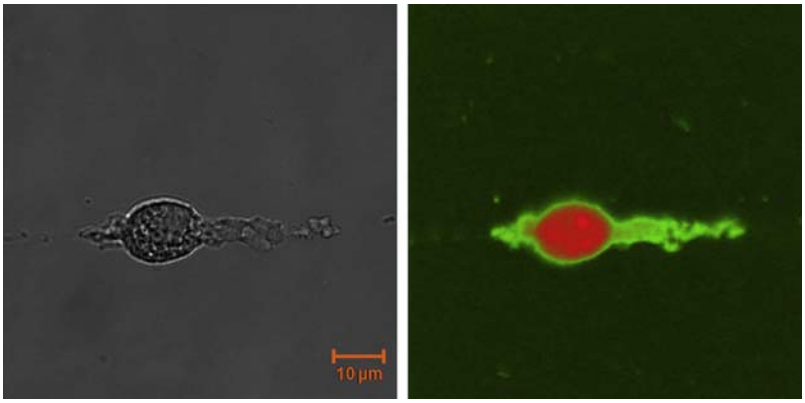

FIGURE 38.5 **Fibroblast encapsulated within a fluorogenic substrate.** Differential interference contrast image (*left*) and fluorescent image (*right*) showing green fluorescence generated by material degradation around cell (*red*).

Biomolecular Factors

In many cases, seeding cells inside a porous scaffold is insufficient to induce tissue regeneration because the material does not contain the chemical cues necessary to promote cellular remodeling events. Thus, researchers have attempted to modify biomaterials actively at the molecular level by incorporating cell-specific biomolecules. One strategy is to encapsulate molecules such as peptides or proteins into biomaterial carriers so that these molecules can be released from the material to trigger or modulate new tissue formation [8]. Another approach involves physically or chemically modifying scaffolds with specific cell-binding peptides to increase cellular interaction with the substrate. Cell-binding peptides are short amino acid sequences derived from much longer native ECM proteins that have been identified as able to incur specific, predictable interactions with cell receptors. Essentially the peptides function to mimic the signaling dynamic between the ECM and cells, and because many synthetic scaffold materials are not inherently adhesive to cells, the introduction of such sequences can be critical to encouraging cell retention and subsequent tissue formation [45]. The most well-studied cell adhesion peptide, arginine-glycine-aspartic acid-serine (RGDS) has been widely used to encourage cells of various types to interact with otherwise nonadhesive synthetic matrices [46] (Fig. 38.6). Other amino acid sequences have been found to promote adhesion by specific cell phenotypes including endothelial cells [29,47–49], smooth muscle cells [47], neural cells [50], and osteoblasts [51].

Various growth factors have also been employed in efforts to enhance the process of histogenesis. Because they have key roles in tissue differentiation and repair, molecules such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor-β1 (TGF-β1) are popular for use in these applications. The release of PDGF from a polyurethane scaffold has been shown to encourage wound healing of in a rat model [52], whereas Griffith and colleagues used tethered EGF to promote osteogenic differentiation in an effort to improve connective tissue regeneration [53]. TGF-β1 has many roles in histogenesis [54] and has received particular attention for aiding the differentiation of stem cells and the development of new vasculature in vivo [55]. In other work, basic fibroblast growth factor (bFGF) and nerve growth factor were immobilized in fibrin scaffolds to facilitate cellular recruitment and differentiation [56]. Biomolecules have also been covalently coupled to PEG-based materials [57–60] with vascular endothelial growth factor and showed the potential to drive endothelial cell tubulogenesis. Furthermore, Delong and West formed gradients of bFGF and observed cellular alignment and migration that was directly influenced by growth factor presentation [61] (Fig. 38.7).

Importance of Microvasculature

One of the biggest limitations to histogenesis in 3D scaffolds is the lack of a functional vascular system. The most successful engineered materials to date have been for tissues such as skin, which are thin enough to be supported by diffusion from the host vascular, and cartilage, which is relatively avascular and as such contains cells that are tolerant of anoxic conditions. All other tissues require some form of vascular system to permit the long-term survival of cells within the material. There are two primary strategies for establishing a blood supply in implanted 3D scaffolds. Vessels can grow into the construct from host tissue or they can be preformed in vitro and interconnect with host vascular upon implantation.

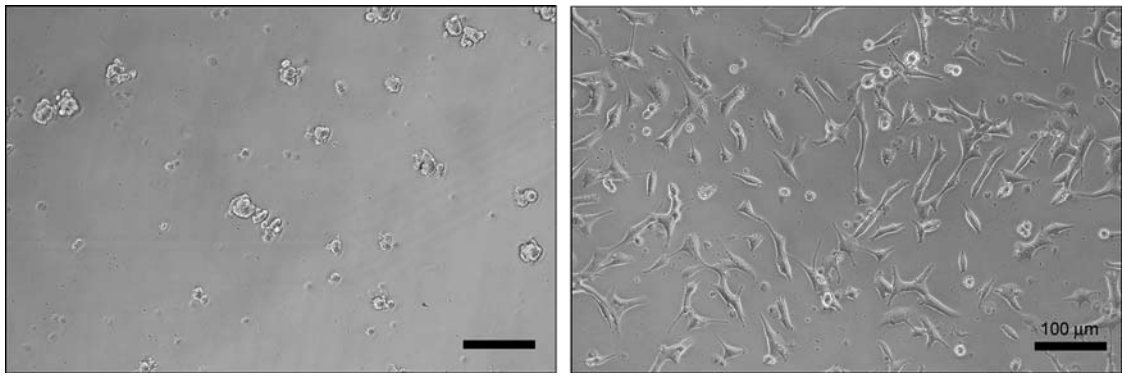

FIGURE 38.6 **Peptide modification promotes cell adhesion.** The nonadhesive nature of polyethylene glycol hydrogels (*left*) can be significantly altered by inclusion of an arginine-glycine-aspartic acid-serine peptide to promote cell attachment (*right*).

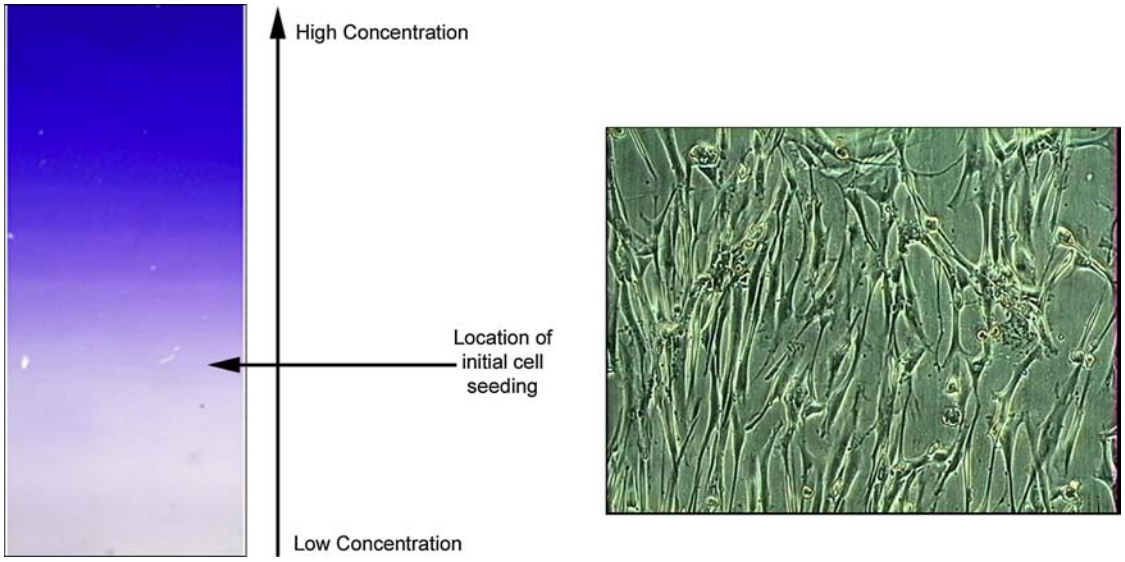

FIGURE 38.7 Gradient of basic fibroblast growth factor (bFGF) immobilized in a hydrogel scaffold. Cells seeded on hydrogels containing a bFGF gradient aligned and migrated along the axis of growth factor immobilization.

The premise behind the first strategy is to encourage vessels to enter an avascular construct from the host tissue by stimulating angiogenesis. To encourage this process, scaffolds can be fabricated with precisely designed pore structures or surface chemistries that support ingrowth [34,62]. In some cases, proangiogenic factors are immobilized on or released from implants to encourage the ingrowth of vessels from host tissue. Work from our laboratory showed extensive infiltration of functional vessels into a cell-adhesive, proteolytically degradable, PEG-based hydrogel that had been implanted in the mouse cornea [63–66]. The limitation of these strategies is the time required for vessels to extend into the entirety of the engineered construct. At an extension rate of approximately 5 μm/h [67], new vessels will not reach the center of large scaffolds until several days after implant, leaving any cells at these locations without sufficient supplies of oxygen and nutrients.

A second option is to create preformed vascular networks in vitro that are capable of anastomosing with host vascular upon implantation. This process of connecting two independent vascular networks is called inosculation and is the mechanism primarily responsible for the successes in plastic surgery and skin transplantations. To generate microvascular networks in vitro, researchers seed scaffolds with cells known to participate in vasculogenesis, including endothelial cells, stem cells, and pericytes. With appropriate biochemical and/or physical stimulation, these cells self-assemble into capillary-like structures. As an example, successful vascular networks have been formed by endothelial cells in fibrin scaffolds [68] and by adult and cord blood–derived progenitor cells in Matrigel [69]. In each study, the preformed vessels were functional upon implantation in vivo; and in the case of the endothelial cells, immature capillaries were further stabilized by host mural cells. Other work along these lines suggests that providing relevant physiomechanical stimulation in vitro will aid in developing functional prevascularized networks in engineered constructs [70–72]. A final approach exploits the body's own ability to form blood vessels. A variety of scaffold materials have been implanted in highly vascularized anatomical sites, where they are incubated for up to 3 weeks as host vessels infiltrate. Once the vessel network is established, the scaffold is explanted, loaded with cells, and finally implanted into the site targeted for regeneration [73].

The need for adequate blood flow in 3D scaffolds is readily apparent and of concern to all researchers interested in regeneration strategies. Advances are promising, although much work remains to be done. Inosculation of preformed microvascular networks with host vascular, for instance, provides functional transport of engineered materials much more rapidly than some other methods, but it is still too slow for sensitive tissue applications. A combination of this approach with proangiogenic strategies may encourage connection in a shorter time, thus leading to the better long-term regeneration of target tissue.

SYNTHETIC MATERIALS FOR HISTOGENESIS OF NEW ORGANS

Biomaterials investigated as scaffolds for histogenesis include natural polymers such as collagen and fibrin, as well as a range of synthetic substrates. Although natural matrices have certain advantages in that their chemical composition is generally amenable to cell growth, batch-to-batch variations in substrate quality and performance can make their use in clinical regeneration applications problematic. As such, the control and flexibility of synthetic materials make them attractive alternatives. As alluded to previously, control is an important element in tailoring the scaffold's material properties for appropriate cell−material and matrix−material interactions. The following sections highlight two popular classes of synthetic materials: hydrolytically degradable polymers and hydrogels.

Hydrolytically Degradable Polymers

The most widely used polymers for cellular scaffold materials are PLA, PGA, or a combination of these two polymers (poly[lactic-*co*-glycolic acid] [PLGA]). PLA, PGA, and PLGA are aliphatic esters that possess good biocompatibility [74] and have been used as drug delivery materials to administer biomolecules during tissue regeneration [75,76]. These polymers are also among the few synthetic polymers approved by the US Food and Drug Administration (FDA) for certain human clinical applications. PGA is extremely hydrophilic in nature; consequently, it will lose its mechanical strength within 2−4 weeks of implantation [77]. PLA, however, contains an additional methyl group and as a result is more hydrophobic. Degradation of PLA scaffolds can take from months to years [75,78]. In addition, the degradation rates of these polymers can be tailored by using copolymer blends (PLGA), which give distinct degradation profiles [75,79]. However, these scaffolds undergo acid-catalyzed hydrolysis and bulk erosion, which have the potential to result in structural instability and interruption of the regeneration process [80].

Polyanhydrides have been synthesized for a number of biomedical applications including tissue engineering and drug delivery [81]. Polyanhydride scaffolds exhibit excellent biocompatibility and contain a large aliphatic component that possesses an ester group that makes the material subject to surface erosion [82]. This deliberate surface erosion is mechanistically different from bulk hydrolysis and can be exploited to synthesize biomaterials scaffolds that have predictable degradation profiles. In addition, the erosion of only the surface of the material allows anhydrides to maintain structural integrity in support of histogenesis. Because they exhibit mechanical properties similar to bone and are ideal scaffolds for tissue infiltration, anhydrides have been widely employed as scaffolds for in vivo bone regeneration [83−85]. Polyanhydride networks can also be combined with other polymers to change their degradation and structural characteristics. Jiang and Zhu [86] showed that anhydride polymers could be polymerized in the presence of PEG to form a cross-linked network with both hydrophobic and hydrophilic components. The hydrophilic PEG chains increase the uptake of water to drive the hydrolysis of the ester bond in the hydrophobic anhydride. As such, the degradation properties can be tailored by altering the amount of PEG in the scaffold material.

Hydrogels

Hydrogels, which contain up to 90% water, are another widely studied class of materials for tissue regeneration. These materials are appealing because the polymer properties are controllable and reproducible [87] and the large water uptake promotes excellent biocompatibility. In many cases, hydrogel mechanical properties resemble those of native tissue and can be systematically controlled for specific applications. In addition, several hydrogel monomers contain vinyl chemical moieties, which are conducive to various free radical−initiated polymerizations schemes that can be employed to generate solid substrate materials. Photoinitiation, for example, enables polymers to be formed using specific wavelengths of light. Using this method, many researchers have had success forming complex 3D structures of varying stiffnesses. For example, polyacrylamide hydrogels have been shown to induce regeneration of soft tissue in facial defects [88], and 2-hydroxyethylmethacrylate has had good success as a fibrillar support for nerve regeneration [89].

Among the most well-studied hydrogel materials is cross-linked PEG, which has been approved by the FDA for use in certain medical applications [90]. As with other hydrogels, the hydrophilic nature of PEG discourages cell and

protein adhesion and therefore results in a low instance of immunorejection by the host. By changing the monomer chain length, adding biological molecules, or using copolymers, researchers have generated a wide array of PEG hydrogel formulations suitable for many different tissue engineering applications. To render an otherwise blank slate amenable to histogenesis, various biomimetic peptides and growth factors have been incorporated into the PEG hydrogel matrix [57,59,91–93]. These modifications have been successful in achieving selective cell adhesion and promoting the accumulation of secreted tissue matrix. As mentioned previously, similar methods have been employed to encourage vasculogenesis and angiogenesis in PEG hydrogel materials. In addition, these hydrogel materials show promise as small-diameter vascular grafts [94]. Incorporation of peptides subject to proteolytic cleavage in the backbone of the PEG monomer renders the scaffolds subject to cell-mediated remodeling, giving these materials an additional advantage as histogenesis conduits.

FUTURE DIRECTIONS IN THREE-DIMENSIONAL SCAFFOLDS: THREE-DIMENSIONAL MICROFABRICATION

Advances in the biomaterial field are providing tissue engineers with the means to generate complex and highly specialized 3D scaffolds. One of the earliest examples of such architectures was developed by Griffith and colleagues for hepatocyte culture and liver regeneration. Using a rapid printing technique, microporous PLGA scaffolds were fabricated by directing solvent streams onto polymer granules in a precisely controlled manner [95]. The hepatocytes seeded upon these constructs exhibited increased metabolic rates that mimicked cells in vivo more closely. In other work, 3D, microporous PLGA foams were prepared by drilling with dies of a specific size. The dimensions of these cylindrical scaffolds were reproducible with millimeter precision, and when placed in vivo, the materials supported bone regeneration in nonhealing defect models [96,97]. Porous scaffolds have also been micropatterned for vascular tissue engineering applications [35,98].

Several researchers have used photopolymerization techniques to mold and pattern hydrogel scaffolds for better control of cell–substrate interactions [99]. Peppas reported micropatterning of PEG hydrogels using UV polymerization to generate many different substrate morphologies on the order of 100 μm [100]. Liu and Bhatia also photopatterned PEG hydrogels using a layer-by-layer method to generate a 3D scaffold for hepatocytes [101].

Laser-based patterning of hydrogels is a relatively new technique for generating complex 3D microenvironments inside hydrogel materials and natural constructs. Liu et al. used a laser ablation technique to form lines, holes, and interconnected grids in collagen matrices [102], whereas growth factors and peptides were patterned by Roy and colleagues using laser-based stereolithography [103] inside a PEG hydrogel. Biomolecules have also been patterned inside agarose hydrogels [104]. In this case, RGDS peptides were patterned in cylindrical shapes within the hydrogel material. After 3 days, neuronal cells seeded on the surface of the materials were shown to have migrated into the hydrogel in only the selectively patterned areas. Additional studies of cell migration in hydrogel materials were conducted with micropatterned, PEG-based materials functionalized with several different bioactive moieties [60,105,106]. In the process of laser scanning lithography, photosensitive peptides or proteins are covalently incorporated into 3D hydrogels with the precision of a confocal microscope laser (Fig. 38.8). The technique is capable of generating features from 1 μm to 1 mm and can be extended to include multiple bioactive moieties in a single substrate. The image in Fig. 38.9 illustrates the 3D nature of a patterned ligand. These precisely fabricated regeneration matrices provide great opportunities for controlled tissue growth.

CONCLUSIONS

The need for replacement tissues and organs is driving tissue engineers to develop materials and strategies capable of generating biologically functional substitutes. The study of natural processes such as wound healing has provided insights into the complex mechanisms of tissue regeneration and allowed researchers to prioritize design parameters for 3D scaffolds. At the same time, advances in biomaterial synthesis and modification, as well as a better understanding of the signaling molecules important in tissue synthesis, are providing a wealth of tools for regeneration strategies. In a systematic approach to histogenesis, Nettles et al. developed a method of neural network analysis in which a self-organizing map delineates the relationships between scaffold parameters, such as cross-link density, and tissue outcomes [107]. The investigators employed this tool with the goal of optimizing and

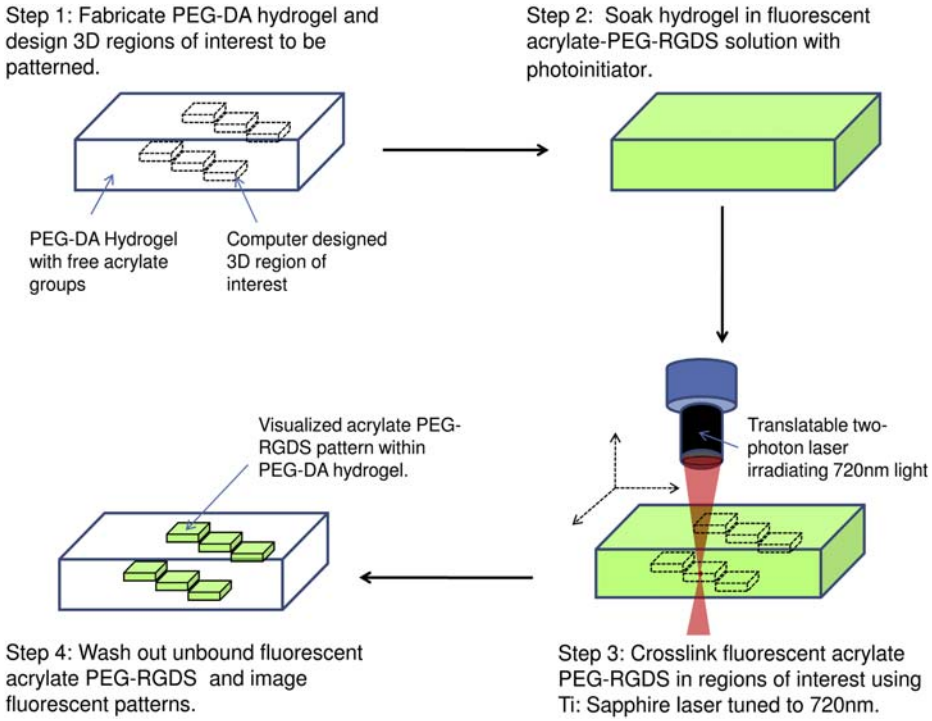

FIGURE 38.8 **Laser scanning lithography patterning of polyethylene glycol (PEG) hydrogels.** Precisely defined patterned areas are generated in three-dimensional (3D) hydrogels by using a confocal microscope laser to cross-link photosensitive materials. *DA*, diacrylate; *RGDS*, arginine-glycine-aspartic acid-serine. *Schematic courtesy of Joseph C. Hoffman, Rice University.*

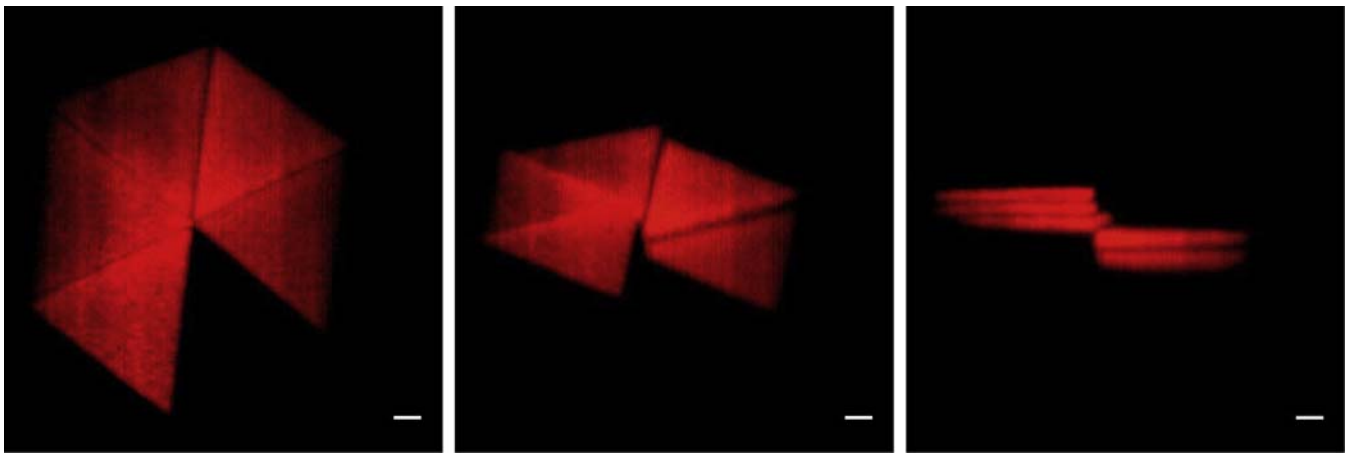

FIGURE 38.9 **Laser scanning lithography pattern of fluorescently labeled arginine-glycine-aspartic acid-serine in a polyethylene glycol hydrogel.** The fluorescent peptide (*red*) is visible in the bulk hydrogel (*black*) after patterning. Scale bar = 10 μm. *Image courtesy of Joseph C. Hoffman, Rice University.*

accelerating the design of a cartilage tissue substitute. Tools such as these will focus the work of tissue engineers going forward. Good success has been seen in developing substitutes for skin and cartilage. Advances in scaffold microvascularization techniques will aid in progressing the field to larger, more complex target tissues.

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CHAPTER

39

Biocompatibility and Bioresponse to Biomaterials

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INTRODUCTION

Biocompatibility is generally defined as the ability of a biomaterial or medical device to perform with an appropriate host response in a specific application. A bioresponse or biocompatibility assessment (i.e., an evaluation of biological responses) is considered to be a measure of the magnitude and duration of the adverse alterations in homeostatic mechanisms that determine the host response. From a practical point of view, the evaluation of biological responses to a medical device is carried out to determine whether the medical device performs as intended and presents no significant harm to the patient. The goal of bioresponse evaluation is to predict whether a biomaterial or medical device presents potential harm to the patient. In regenerative medicine, biomaterials are used in a wide variety of ways ranging from carriers of genetic material to tissue-engineered implants that may contain autologous, allogeneic, or xenogeneic genetic materials, cells, and scaffold materials. Scaffolds may be composed of synthetic or modified-natural materials. A tissue-engineered implant is a biological–biomaterial combination in which some component of tissue has been combined with a biomaterial to create a device to restore or modify tissue or organ function. Thus, tissue-engineered devices with a biological component(s) require an expanded perspective and understanding of biocompatibility and biological response evaluation. The purpose of this chapter is to provide an overview of this expanded perspective. Each unique tissue-engineered device requires a distinctive set of experiments to determine its biological responses and biocompatibility.

This chapter presents an overview of host responses that must be considered in determining the biocompatibility of tissue-engineered devices that employ biomaterials. The three major responses that must be considered for biocompatibility assessment are inflammation, wound healing, and immunological reactions or immunity. For the purposes of biological response evaluation, immunological reactions or immunity are considered to be immunotoxicity.

Pathologists use the terminology of inflammation and immunity to describe adverse tissue reactions, whereas immunologists commonly refer to inflammation as innate immunity and activation of the immune system as being acquired immunity. Tissue–material interactions are a series of responses that are initiated by the implantation procedure, as well as by the presence of the biomaterial, medical device, or tissue-engineered device. In this chapter, we divide the series of tissue–material responses into inflammation (innate immunity) and wound healing, and immunotoxicity. After implantation, early, transient tissue–material responses include injury (implantation), blood–materials interactions, provisional matrix formation, and the temporal sequence of inflammation and wound healing including acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction, and ultimately fibrosis or fibrous capsule (scar) development. Immunotoxicity is any adverse effect on the function or structure of the immune system or other systems as a result of an immune system dysfunction. Two significant failure mechanisms of tissue-engineered devices are fibrosis or fibrous capsule (scar) development surrounding and infiltrating the tissue-engineered device, or the initiation of acquired or cellular immunity by the biological component of the tissue-engineered device. Also, the biological component and the biomaterial component in a tissue-engineered device may act in concert or synergistically to facilitate either of these failure mechanisms.

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INFLAMMATION (INNATE IMMUNITY) AND WOUND HEALING

The process of implanting a biomaterial or tissue-engineered device results in injury to tissues or organs [1–8]. This injury and the subsequent perturbation of homeostatic mechanisms lead to inflammatory responses, foreign body reaction, and wound healing. The response to injury depends on multiple factors that include the extent of injury, loss of basement membrane structures, blood–material interactions, provisional matrix formation, the extent or degree of cellular necrosis, and the extent of the inflammatory response. The organ or tissue undergoing implantation may have a significant role in the response. These events, in turn, may affect the extent or degree of granulation tissue formation, foreign body reaction, and fibrosis or fibrous capsule (scar) development [9]. These events are summarized in Table 39.1. These host reactions for biocompatible biomaterials are considered to be normal. They are also tissue-, organ-, and species-dependent. These dependencies thus provide perspectives on the biological response evaluation and the ultimate determination of biocompatibility. The reactions occur or are initiated early (that is, within 2–3 weeks of the time of implantation) and undergo resolution quickly, leading to fibrosis or fibrous capsule formation.

Blood–Material Interactions and Initiation of the Inflammatory Response

Blood–material interactions and the inflammatory response are intimately linked; in fact, early responses to injury involve mainly blood and the vasculature [1–8]. Regardless of the tissue into which a biomaterial is implanted, the initial inflammatory response is activated by injury to vascularized connective tissue. Because blood and its components are involved in the initial inflammatory responses, thrombus, blood clot, or both also form. Thrombus formation involves activation of the extrinsic and intrinsic coagulation systems, the complement system, the fibrinolytic system, the kinin-generating system, and platelets. Thrombus or blood clot formation on the surface of a biomaterial is related to the well-known Vroman effect of protein adsorption. From a wound healing perspective, blood protein deposition on a biomaterial surface is described as provisional matrix formation.

Although injury initiates the inflammatory response, released chemicals from plasma, cells, and injured tissue mediate the response [4,6,10,11]. Important classes of chemical mediators of inflammation are presented in Table 39.2. Several important points must be noted to understand the inflammatory response and how it relates to biomaterials. First, although chemical mediators are classified on a structural or functional basis, different mediator systems interact and provide a system of checks and balances regarding their respective activities and functions. Second, chemical mediators are quickly inactivated or destroyed, which suggests that their action is predominantly local (i.e., at the implant site). Third, generally acid, lysosomal proteases, and oxygen-derived free radicals produce the most significant damage or injury. These chemical mediators are also important in the degradation of biomaterials. Phagolysosomes in macrophages can have acidity as low as pH 4, and direct microelectrode studies of this acidic environment have determined pH levels to be as low as 3.5. Moreover, only several hours are necessary to achieve these acid levels after adhesion of macrophages [12–14].

The predominant cell type present in the inflammatory response varies with the age of the injury. In general, neutrophils, commonly called polymorphonuclear leukocytes or polys, predominate during the first several days after injury and then are replaced by monocytes as the predominant cell type. Three factors account for this change in cell type [1]: Neutrophils are short-lived and disintegrate and disappear after 24–48 h; neutrophil emigration is of

TABLE 39.1 Sequence of Host Reactions

Injury
Blood–material interactions
Provisional matrix formation
Acute inflammation
Chronic inflammation
Granulation tissue
Foreign body reaction
Fibrosis/fibrous capsule development

TABLE 39.2 Important Chemical Mediators of Inflammation Derived From Plasma, Cells, or Injured Tissue

Mediators	Examples
Vasoactive agents	Histamine, serotonin, adenosine, endothelial-derived relaxing factor, prostacyclin, endothelin, thromboxane a_2
Plasma proteases	
Kinin system	Bradykinin, kallikrein
Complement system	C3a, C5a, C3b, C5b–C9
Coagulation/fibrinolytic system	Fibrin degradation products, activated Hageman factor (FXIIA), tissue plasminogen activator
Leukotrienes	Leukotriene B_4, hydroxyeicosatetraenoic acid
Lysosomal proteases	Collagenase, elastase
Oxygen-derived free radicals	H_2O_2, superoxide anion, nitric oxide
Platelet-activating factors	Cell membrane lipids
Cytokines	Interleukin-1, tumor necrosis factor
Growth factors	Platelet-derived growth factor, fibroblast growth factor, transforming growth factor-α or β, epithelial growth factor

short duration because chemotactic factors for neutrophil migration are activated early in the inflammatory response [2]. After emigration from the vasculature, monocytes differentiate into macrophages, and these cells are very long-lived (up to months) [3]. Monocyte emigration may continue for days to weeks, depending on the injury and implanted biomaterial, and chemotactic factors for monocytes are activated over longer periods of time.

Provisional Matrix Formation

Injury to vascularized tissue in the implantation procedure leads to immediate development of the provisional matrix at the implant site. This provisional matrix consists of fibrin, produced by activation of the coagulative and thrombosis systems, and inflammatory products released by the complement system, activated platelets, inflammatory cells, and endothelial cells [15–18]. These events occur early, within minutes to hours after implantation of a medical device. Components within or released from the provisional matrix (that is, fibrin network [thrombosis or clot]) initiate the resolution, reorganization, and repair processes such as inflammatory cell and fibroblast recruitment. Platelets activated during the fibrin network formation release platelet factor 4, platelet-derived growth factor (PDGF), and transforming growth factor β (TGF-β), which contribute to fibroblast recruitment [19,20]. Upon activation, monocytes and lymphocytes generate additional chemotactic factors including leukotriene B_4 (LTB_4), PDGF, and TGF-β to recruit fibroblasts.

The provisional matrix is composed of adhesive molecules such as fibronectin and thrombospondin bound to fibrin as well as platelet granule components released during platelet aggregation. Platelet granule components include thrombospondin, released from the platelet α-granule, and cytokines including TGF-α, TGF-β, PDGF, platelet factor 4, and platelet-derived endothelial cell growth factor. The provisional matrix is stabilized by the cross-linking of fibrin by factor XIIIa.

The provisional matrix appears to provide both structural and biochemical components to the process of wound healing. The complex three-dimensional structure of the fibrin network with attached adhesive proteins provides a substrate for cell adhesion and migration. The presence of mitogens, chemoattractants, cytokines, and growth factors within the provisional matrix provides for a rich milieu of activating and inhibiting substances for various cellular proliferative and synthetic processes.

The provisional matrix may be viewed as a naturally derived, biodegradable, sustained release system in which mitogens, chemoattractants, cytokines, and growth factors are released to control subsequent wound healing processes [21–27]. Despite the rapid increase in our knowledge of the provisional matrix and its capabilities, our knowledge of the control of the formation of the provisional matrix and its effect on subsequent wound healing events is poor.

Temporal Sequence of Inflammation and Wound Healing

Inflammation is generally defined as the reaction of vascularized living tissue to local injury. Inflammation serves to contain, neutralize, dilute, or wall off the injurious agent or process. In addition, it sets into motion a series of events that may heal and reconstitute the implant site by replacing the injured tissue by regenerating native parenchymal cells, forming fibroblastic scar tissue, or a combination of these processes [4,6].

The sequence of events after implantation of a biomaterial is illustrated in Fig. 39.1. The size, shape, and chemical and physical properties of the biomaterial and the physical dimensions and properties of the prosthesis or device may be responsible for variations in the intensity and time duration of the inflammatory and wound healing processes. Thus, the intensity and/or time duration of the inflammatory reaction may characterize the biocompatibility of a biomaterial or device.

Classically, the biocompatibility of an implanted material has been described in terms of the morphological appearance of the inflammatory reaction to the material; however, the inflammatory response is a series of complex reactions involving various types of cells, the densities, activities, and functions of which are controlled by various endogenous and autacoid mediators. The simplistic view of the acute inflammatory response progressing to the chronic inflammatory response may be misleading with respect to biocompatibility studies and the inflammatory response to implants. In vivo studies using the cage implant system show that monocytes and macrophages are present in highest concentrations when neutrophils are also at their highest concentrations: that is, the acute inflammatory response [28,29]. Neutrophils have short lifetimes (hours to days) and disappear from the exudates more rapidly than do macrophages, which have lifetimes of days to weeks to months. Eventually, macrophages become the predominant cell type in the exudates, resulting in a chronic inflammatory response. Monocytes rapidly differentiate into macrophages, the cells principally responsible for normal wound healing in the foreign body reaction. Classically, the development of granulation tissue has been considered to be part of chronic inflammation, but because of unique tissue–material interactions, it is preferable to differentiate the foreign body reaction, with its varying degree of granulation tissue development, including macrophages, fibroblasts, and capillary formation, from chronic inflammation.

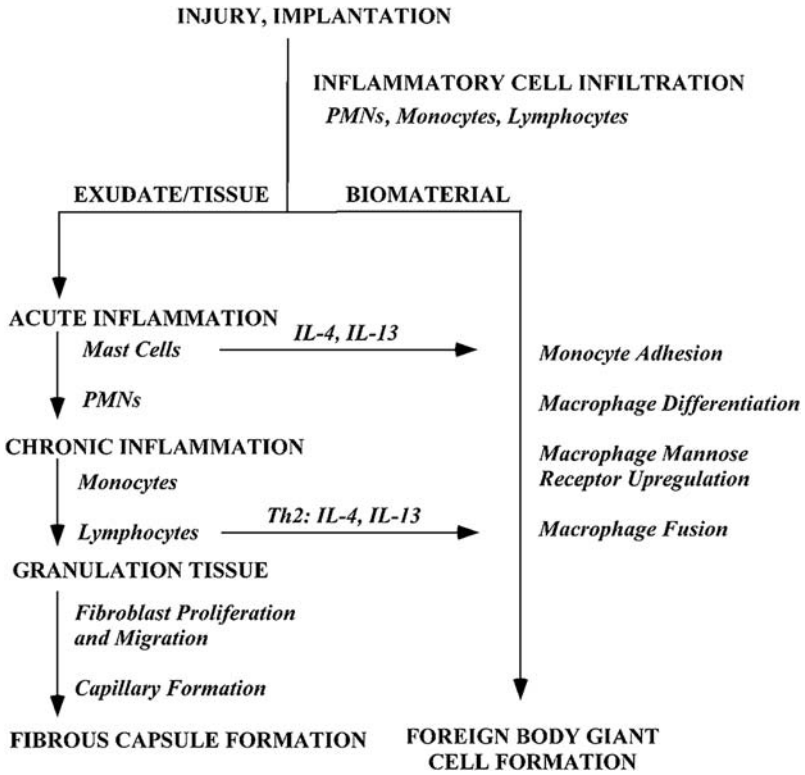

FIGURE 39.1 **Sequence of events involved in inflammatory and wound healing responses leading to foreign body giant cell (FBGC) formation.** This shows the potential importance of mast cells in the acute inflammatory phase and T-helper 2 (Th2) lymphocytes in the transient chronic inflammatory phase with the production of interleukin (IL)-4 and IL-13, which can induce monocyte/macrophage fusion to form FBGCs. *PMN*, polymorphonuclear.

Acute Inflammation

Acute inflammation is of relatively short duration, lasting from minutes to days, depending on the extent of injury. The main characteristics of acute inflammation are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes (predominantly neutrophils). Neutrophils and other motile white cells emigrate or move from the blood vessels to the perivascular tissues and the injury (implant) site [30–32].

The accumulation of leukocytes, in particular neutrophils and monocytes, is the most important feature of the inflammatory reaction. Leukocytes accumulate through a series of processes including margination, adhesion, emigration, phagocytosis, and extracellular release of leukocyte products [33]. Increased leukocytic adhesion in inflammation involves specific interactions between complementary "adhesion molecules" present on the leukocyte and endothelial surfaces [34,35]. The surface expression of these adhesion molecules is modulated by inflammatory agents; mechanisms of interaction include stimulation of leukocyte adhesion molecules (C5a and LTB_4), stimulation of endothelial adhesion molecules (interleukin-1 [IL-1]), or both effects of tumor necrosis factor-α (TNF-α). Integrins are composed of a family of transmembrane glycoproteins that modulate cell–matrix and cell–cell relationships by acting as receptors to extracellular protein ligands and also as direct adhesion molecules [36]. An important group of integrins (adhesion molecules) on leukocytes include the CD11/CD18 family of adhesion molecules. Inflammatory mediators (i.e., cytokines) stimulate a rapid increase in these adhesion molecules on the leukocyte surface as well as increased leukocyte adhesion to endothelium. Leukocyte–endothelial cell interactions are also controlled by endothelial–leukocyte adhesion molecules (also known as E-selectins) or intracellular adhesion molecules (ICAMs) such as ICAM-1 and ICAM-2, and vascular cell adhesion molecules on endothelial cells [37].

Inflammatory cell emigration is controlled in part by chemotaxis, which is the unidirectional migration of cells along a chemical gradient. A wide variety of exogenous and endogenous substances have been identified as chemotactic agents [11,30–41]. Important to the emigration or movement of leukocytes is the presence of specific receptors for chemotactic agents on the cell membranes of leukocytes. These and other receptors also may have a role in activating leukocytes. After localization of leukocytes at the injury (implant) site, phagocytosis and the release of enzymes occur following activation of neutrophils and macrophages. The major role of the neutrophils in acute inflammation is to phagocytose microorganisms and foreign materials. Phagocytosis is seen as a three-step process in which the injurious agent undergoes recognition and neutrophil attachment, engulfment, and killing or degradation. With regard to biomaterials, engulfment and degradation may or may not occur, depending on the properties of the biomaterial.

Although biomaterials are not generally phagocytosed by neutrophils or macrophages because of the size disparity (i.e., the surface of the biomaterial is greater than the size of the cell), certain events may occur in phagocytosis. The process of recognition and attachment is expedited when the injurious agent is coated by naturally occurring serum factors called opsonins. The two major opsonins are immunoglobulin (Ig)G and the complement-activated fragment, C3b. Both of these plasma-derived proteins are known to adsorb to biomaterials, and neutrophils and macrophages have corresponding cell membrane receptors for these opsonization proteins. These receptors may also have a role in activating the attached neutrophil or macrophage. Because of the size disparity between the biomaterial surface and the attached cell, "frustrated phagocytosis" may occur [38,39]. This process does not involve engulfing the biomaterial but it causes the extracellular release of leukocyte products in an attempt to degrade the biomaterial. Neutrophils adherent to complement-coated and Ig-coated nonphagocytosable surfaces may release enzymes by direct extrusion or exocytosis from the cell [38,39]. The amount of enzyme released during this process depends on the size of the polymer particle, with larger particles inducing greater amounts of enzyme release. This suggests that the specific mode of cell activation in the inflammatory response in tissue depends on the size of the implant and that a material in a phagocytosable form (e.g., powder or particulate) may provoke a degree of inflammatory response different from that of the same material in a nonphagocytosable form (e.g., film).

Tissue-engineered constructs containing biomaterial scaffolds alone or with cells and/or chemokines, growth factors, or other biological components are thus subjected to an aggressive microenvironment that may quickly compromise the intended function of the construct [42].

Chronic Inflammation

Chronic inflammation is less uniform histologically than is acute inflammation. In general, chronic inflammation is characterized by the presence of monocytes and lymphocytes with the early proliferation of blood vessels and connective tissue [4,6,43–45]. Many factors modify the course and histological appearance of chronic inflammation.

Persistent inflammatory stimuli lead to chronic inflammation. Although the chemical and physical properties of the biomaterial may lead to chronic inflammation, motion in the implant site by the biomaterial may also produce chronic inflammation. The chronic inflammatory response to biomaterials is confined to the implant site. Inflammation with the presence of mononuclear cells, including lymphocytes and plasma cells, is given the designation chronic inflammation, whereas the foreign body reaction with granulation tissue development is considered the normal wound healing response to implanted biomaterials (i.e., the normal foreign body reaction). Chronic inflammation with biocompatible materials is usually of short duration (i.e., a few days).

Lymphocytes and plasma cells are involved principally in immune reactions and are important mediators of antibody production and delayed hypersensitivity responses. Their roles in nonimmunological injuries and inflammation are largely unknown [46–48]. Little is known regarding immune responses and cell-mediated immunity to synthetic biomaterials. The role of macrophages must be considered in the possible development of immune responses to synthetic biomaterials. Macrophages and dendritic cells process and present the antigen to immunocompetent cells and thus are critical mediators in the development of immune reactions.

The macrophage is probably the most important cell in chronic inflammation because of the great number of biologically active products it produces [44]. Important classes of products yielded and secreted by macrophages include neutral proteases, chemotactic factors, arachidonic acid metabolites, reactive oxygen metabolites; complement components, coagulation factors, growth-promoting factors, and cytokines [49–51].

Growth factors such as PDGF, fibroblast growth factor, TGF-β, TGF-α/epithelial growth factor, and IL-1 or TNF are important to the growth of fibroblasts and blood vessels and the regeneration of epithelial cells. Growth factors released by activated cells stimulate the production of a wide variety of cells; initiate cell migration, differentiation, and tissue remodeling; and may be involved in various stages of wound healing [19,52–56]. There is a lack of information regarding interaction and synergy among various cytokines and growth factors and their abilities to exhibit chemotactic, mitogenic, and angiogenic properties.

Granulation Tissue

Within 1 day after implantation of a biomaterial (i.e., injury), the healing response is initiated by the action of monocytes and macrophages, followed by the proliferation of fibroblasts and vascular endothelial cells at the implant site, leading to the formation of granulation tissue, the hallmark of healing inflammation. Granulation tissue derives its name from the pink, soft, granular appearance on the surface of healing wounds; its characteristic histological features include the proliferation of new small blood vessels and fibroblasts. Depending on the extent of injury, granulation tissue may be seen as early as 3–5 days after the implantation of a biomaterial.

The new small blood vessels are formed by budding or sprouting of preexisting vessels in a process known as neovascularization or angiogenesis [45,57–60]. This process involves proliferation, maturation, and organization of endothelial cells into capillary tubes. Fibroblasts also proliferate in developing granulation tissue and are active in synthesizing collagen and proteoglycans. In the early stages of granulation tissue development, proteoglycans predominate; later, however, collagen (especially type I collagen) predominates and forms the fibrous capsule. Some fibroblasts in developing granulation tissue may have features of smooth muscle cells. These cells are called myofibroblasts and are considered to be responsible for the wound contraction seen during the development of granulation tissue.

Macrophage Interactions

The inflammatory and immune systems overlap considerably through the activity and phenotypic expression of macrophages that are derived from blood-borne monocytes. Monocytes and macrophages belong to the mononuclear phagocytic system (MPS) (Table 39.3). Cells in the MPS may be considered resident macrophages in the respective tissues that take on specialized functions that depend on their tissue environment. From this perspective, the host defense system may be seen as blood-borne or circulating inflammatory and immune cells as well as mononuclear phagocytic cells that reside in specific tissues with specialized functions. In the inflammatory and immune responses, the macrophage has a pivotal role in both the induction and effector phases of these responses.

Two factors that have a role in monocyte or macrophage adhesion and activation and foreign body giant cell (FBGC) formation are the surface chemistry of the substrate onto which the cells adhere and the protein adsorption that occurs before cell adhesion. These two factors have been hypothesized to have significant roles in the inflammatory and wound healing responses to biomaterials and medical devices in vivo.

TABLE 39.3 Mononuclear Phagocytic System

Tissues	Cells
Implant sites	Inflammatory macrophages, foreign body giant cells
Liver	Kupffer cells
Lung	Alveolar macrophages
Connective tissue	Histiocytes
Bone marrow	Macrophages
Spleen and lymph nodes	Fixed and free macrophages
Serous cavities	Pleural and peritoneal macrophages
Nervous system	Microglial cells
Bone	Osteoclasts
Skin	Langerhans cells and dendritic cells
Lymphoid tissue	Dendritic cells

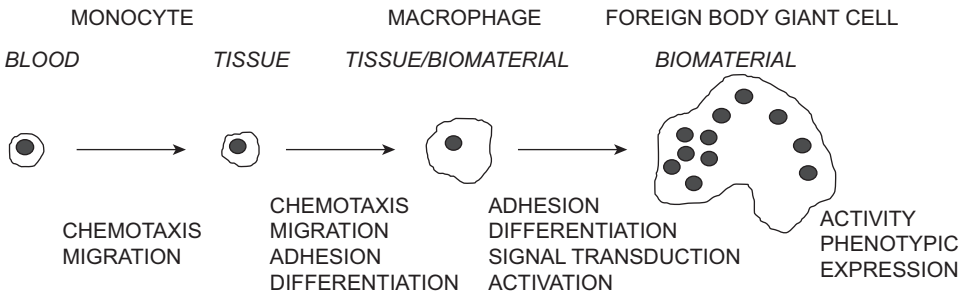

FIGURE 39.2 **In vivo transition from blood-borne monocyte to biomaterial-adherent monocyte/macrophage to foreign body giant cell (FBGC) at the tissue–biomaterial interface.** Little is known regarding the indicated biological responses that are considered to have important roles in the transition to FBGC development.

Macrophage interactions with biomaterials are initiated when blood-borne monocytes in the early transient responses migrate to the implant site and adhere to the blood protein adsorbed biomaterial through monocyte–integrin interactions. After adhesion, adherent monocytes differentiate into macrophages that then may fuse to form FBGCs. Fig. 39.2 demonstrates the progression from circulating blood monocyte to tissue macrophage to FBGC development that is most commonly observed. Because of the progression of monocytes to macrophages to FBGCs (Fig. 39.2), the following discussion of macrophage interactions includes perspectives on how macrophages are formed (i.e., monocyte adhesion) and what happens to macrophages on biomaterial surfaces (i.e., FBGC formation) [61,62].

Material surface property-dependent blood protein adsorption occurs immediately upon surgical implantation of a biomaterial; it is the protein-modified biomaterial that inflammatory cells subsequently encounter. Monocytes express receptors for various blood components, but they recognize naturally occurring foreign surfaces by receptors for opsonins such as fragments of complement component C3. Complement activation by biomaterials has been well-documented [63]. Exposure to blood during biomaterial implantation may permit extensive opsonization with the labile fragment C3b and the rapid conversion of C3b to its hemolytically inactive but nevertheless opsonic and more stable form, C3bi. C3b is bound by the CD35 receptor, but C3bi is recognized by distinct receptors, CD11b/CD18 and CD11c/CD18, on monocytes [61]. Fibrinogen, a major plasma protein that adsorbs to biomaterials, is another ligand for these receptors that, together with CD11a/CD18, constitutes a subfamily of integrins that is restricted to leukocytes [61,62]. Studies with monoclonal antibodies to their common β_2 subunit (CD18) and distinct α-chains have implicated CD11b/CD18 and CD11c/CD18 in monocyte/macrophage responses. Other potential adhesion-mediating proteins that adsorb to biomaterials include IgG, which may interact with monocytes via various receptors and fibronectin, for which monocytes also express multiple types of receptors [64–67].

Foreign Body Giant Cell Formation and Interactions

The foreign body reaction is composed of FBGCs and the components of granulation tissue, which consist of macrophages, fibroblasts, and capillaries in varying amounts, depending on the form and topography of the implanted material [68]. Relatively flat and smooth surfaces, such as those found on breast prostheses, have a foreign body reaction that is composed of a layer of macrophages one to two cells thick. Relatively rough surfaces, such as those found on the outer surfaces of expanded poly(tetrafluoroethylene) vascular prostheses or poly(methyl methacrylate) bone cement, have a foreign body reaction composed of several layers of macrophages and FBGCs at the surface. Fabric materials generally have a surface response composed of macrophages and FBGCs with varying degrees of granulation tissue subjacent to the surface response.

As previously discussed, the form and topography of the surface of the biomaterial determine the composition of the foreign body reaction. With biocompatible materials, the composition of the foreign body reaction in the implant site may be controlled by the surface properties of the biomaterial, the form of the implant, and the relationship between the surface area of the biomaterial and the volume of the implant. For example, high surface-to-volume implants such as fabrics or porous materials will have higher ratios of macrophages and FBGCs in the implant site than will smooth-surface implants, which will have fibrosis as a significant component of the implant site.

The foreign body reaction consisting mainly of macrophages and/or FBGCs may persist at the tissue−implant interface for the lifetime of the implant [1,2,69−71]. Generally, fibrosis (i.e., fibrous encapsulation) surrounds the biomaterial or implant with its interfacial foreign body reaction, isolating the implant and foreign body reaction from the local tissue environment. Early in the inflammatory and wound healing response, the macrophages are activated upon adherence to the material surface [50]. Although the chemical and physical properties of the biomaterial are generally considered to be responsible for macrophage activation, the nature of subsequent events regarding the activity of macrophages at the surface is not clear. Tissue macrophages, derived from circulating blood monocytes, may coalesce to form multinucleated FBGCs. FBGCs containing large numbers of nuclei are typically present on the surface of biomaterials. Although these FBGCs may persist for the lifetime of the implant, it is not known whether they remain activated, releasing their lysosomal constituents, or become quiescent. FBGCs have been implicated in the biodegradation of polymeric medical devices [72−74].

Fig. 39.1 demonstrates the sequence of events involved in inflammation and wound healing when medical devices are implanted. In general, the neutrophil (polymorphonuclear) predominant acute inflammatory response and the lymphocyte/monocyte predominant chronic inflammatory response resolve quickly (i.e., within 2 weeks), depending on the type and location of the implant. Studies using IL-4 demonstrate the role for type 2 (Th2) helper lymphocyte and mast cells in the development of the foreign body reaction at the tissue−material interface [75]. Th2 helper lymphocytes have been described as "antiinflammatory" based on their cytokine profile, of which IL-4 is a significant component. Th2 helper lymphocytes also produce IL-13, which has an effect similar to that of IL-4 on FBGC formation. In this regard, anti-IL-4 antibody does not inhibit IL-13−induced FBGC formation, nor does anti-IL-13 antibody inhibit IL-4−induced FBGC formation. In IL-4 and IL-13 FBGC culture systems, the macrophage mannose receptor (MMR) has been identified as critical to the fusion of macrophages in the formation of FBGC [76,77]. FBGC formation can be prevented by competitive inhibitors of MMR activity (i.e., α-mannan) or inhibitors of glycoprotein processing that restrict MMR surface expression.

Regarding the effect of lymphocytes on the foreign body reaction, studies have demonstrated that interactions with lymphocytes enhance adherent macrophage and FBGC production of proinflammatory cytokines. Interactions through indirect (paracrine) signaling showed a significant proinflammatory effect in enhancing adherent macrophage/FBGC at early time points, whereas interactions via direct (juxtacrine) mechanisms dominated at later points. Furthermore, lymphocytes prefer interactions with adherent macrophages and FBGCs, as opposed to biomaterial surfaces, resulting in lymphocyte activation [78−81]. In vivo studies using clinically synthetic biomaterials have demonstrated that there is a quantitative increase in T cells after secondary biomaterial exposure, but the T-cell subset distribution does not change, indicating nonspecific recruitment of T cells rather than an adaptive immune response. Studies in T cell−deficient mice have shown no change in foreign body giant cell formation. In vitro studies with clinical synthetic biomaterials showed no expression of the activation markers CD69 and CD25 and lymphocyte proliferation was not identified [82−85]. Results from these in vivo and in vitro studies do not suggest an adaptive immune response with clinically relevant biomaterials, because T cell markers CD25 and CD69 were not upregulated and T cell cytokines IL-2 and interferon-gamma were not present.

FBGC formation at the surface of implanted biomaterials occurs quickly and within days to weeks after implantation. Fig. 39.3 shows scanning electron micrographs of monocytes, macrophages, and FBGCs at various times after implantation of a biocompatible polyurethane material in the in vivo rat cage system. Monocyte adhesion

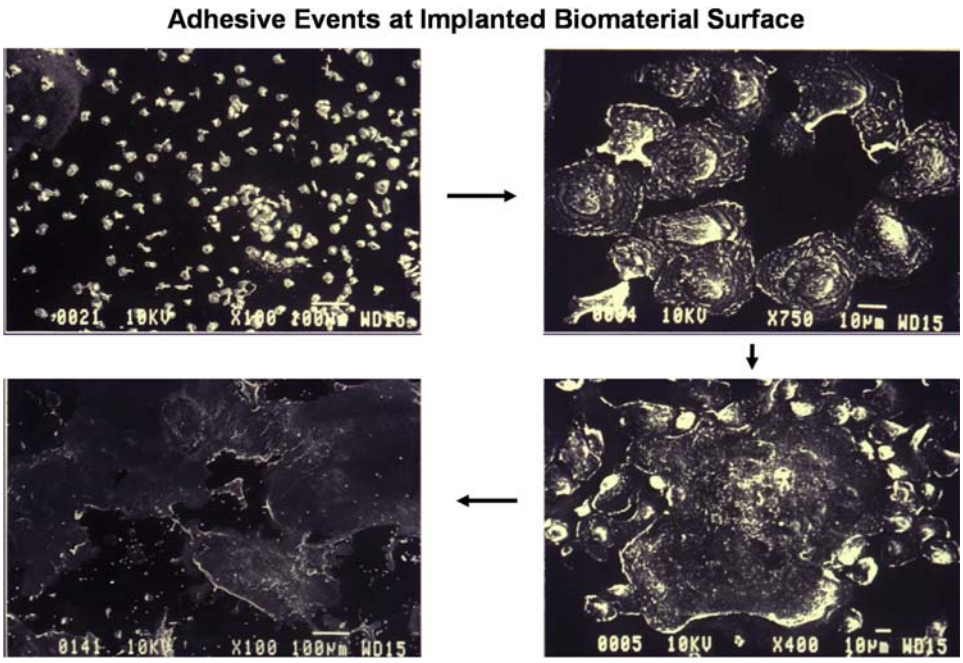

FIGURE 39.3 **In vivo time-dependent processes of monocyte adhesion (A), macrophage development (B), early macrophage fusion to form foreign body giant cells (C), and late macrophage and foreign body giant cell fusion to form large foreign body giant cells (D).**

(Fig. 39.3A) occurs quickly, at 2–4 days. Macrophage development is seen within 1 week after implantation (Fig. 39.3B). Macrophage fusion to form FBGCs can occur within 1–2 weeks after implantation (Fig. 39.3C); interestingly, FBGCs themselves can fuse to produce exceptionally large giant cells on the surface (Fig. 39.3D). Of interest in the use of porous scaffolds and other forms of topographical variations of implanted tissue-engineered systems, these large giant cells may be significantly large enough to cover the porous structure and thus inhibit cellular infiltration into the respective pores as well as inhibit diffusion from these porous structures. The formation of large FBGCs formed late (i.e., weeks) may compromise the function of tissue-engineered medical devices in which porosity and diffusion are important design criteria for the function of the device. Examples of such behavior are the development of biosensors and of complex tissue-engineered scaffolds in which proteins and other important modifying agents depend on diffusion from the device into the microenvironment. The potential for these events should be considered in the design of in vivo biocompatibility studies.

FIBROSIS AND FIBROUS ENCAPSULATION

The end-stage healing response to biomaterials is generally fibrosis or fibrous encapsulation. However, tissue-engineered devices may be exceptions to this general statement (e.g., porous materials inoculated with parenchymal cells or porous materials implanted into bone).

Repair of implant sites involves two distinct processes: regeneration, which is the replacement of injury tissue by parenchymal cells of the same type, or replacement by connective tissue that constitutes the fibrous capsule. These processes are generally controlled by either the proliferative capacity of the cells in the tissue receiving the implant and the extent of injury as it relates to the destruction or persistence of the tissue framework of the implant site. The regenerative capacity of cells permits classification into three groups: labile, stable (or expanding), and permanent (or static) cells. Labile cells continue to proliferate throughout life, stable cells retain this capacity but do not normally replicate, and permanent cells cannot reproduce themselves after birth. Perfect repair with restitution of normal structure theoretically occurs only in tissue consisting of stable and labile cells, whereas all injuries to tissues composed of permanent cells may result in fibrosis and fibrous capsule formation with little restitution of the normal tissue or organ structure. Tissues composed of permanent cells (e.g., nerve cells, skeletal muscle cells, and cardiac muscle cells) most commonly undergo an organization of the inflammatory exudates, leading to fibrosis. Tissues composed of stable cells (e.g., parenchymal cells of the liver, kidney, and pancreas), mesenchymal cells

(e.g., fibroblasts, smooth muscle cells, osteoblasts, and chondroblasts), and vascular endothelial and labile cells (e.g., epithelial cells and lymphoid and hematopoietic cells) may also follow this pathway to fibrosis or may undergo resolution of the inflammatory exudates, leading to restitution of the normal tissue structure. The condition of the underlying framework or supporting stroma of the parenchymal cells after an injury has an important role in restoring normal tissue structure. Retention of the framework may lead to restitution of the normal tissue structure, whereas destruction of the framework most commonly leads to fibrosis. It is important to consider the species-dependent nature of the regenerative capacity of cells. For example, cells from the same organ or tissue but from different species may exhibit different regenerative capacities and/or connective tissue repair.

The extent of provisional matrix formation is an important factor because it is related to wound healing by first or second intention. First intention (primary union) wound healing occurs when there is minimal to no space between the tissue and device, whereas second intention (secondary union) wound healing occurs when a large space is present, providing for extensive provisional matrix formation. Obviously, inappropriate or inadequate preparation of the implant site leading to extensive provisional matrix formation may predispose the implant to failure through mechanisms related to fibrous capsule formation.

The inflammatory (innate) and immune (adaptive) responses have common components. It is possible to have inflammatory responses only with no adaptive immune response. In this situation, both humoral and cellular components that are shared by both types of responses may only participate in the inflammatory response. Table 39.4 indicates the common components of the inflammatory (innate) and immune (adaptive) responses. Macrophages and dendritic cells are known as professional antigen-presenting cells responsible for the initiation of the adaptive immune response.

Many investigators have considered macrophages and dendritic cells to be distinctly different antigen-presenting cells (APCs). Hume summarized evidence that dendritic cells are part of the mononuclear phagocyte system and are derived from the same common macrophage precursor; they are responsive to the same growth factors, express the same surface markers, and have no unique adaptation for antigen presentation that is not shared by other macrophages [86].

IMMUNOTOXICITY (ACQUIRED IMMUNITY)

The acquired or adaptive immune system acts to protect the host from foreign agents or materials and usually is initiated through specific recognition mechanisms and the ability of humoral and cellular components to recognize the foreign agent or material as being "nonself" [18,87–92]. Generally, the adaptive immune system may be considered to have two components: humoral or cellular. Humoral components include antibodies, complement components, cytokines, chemokines, growth factors, and other soluble mediators. These components are synthesized by cells of the immune response; in turn, they function to regulate the activity of these cells and provide for communication between different cells in the cellular component of the adaptive immune response. Cells of the immune system arise from stem cells in the bone marrow (B lymphocytes) or the thymus (T lymphocytes) and differ from each other in morphology, function, and the expression of cell-surface antigens. They share the common features of maintaining cell-surface receptors that assist in recognizing and/or eliminating foreign materials. Regarding tissue-engineered devices, the adaptive immune response may recognize the biological components, modifications

TABLE 39.4 Common Components of Inflammatory (Innate) and Immune (Adaptive) Responses

Components
Complement cascade components
Immunoglobulins
Cellular components
Macrophages
Natural killer cells
Dendritic cells
Cells with dual phagocytic and antigen-presenting capabilities

of the biological components, or degradation products of the biological components, commonly known as antigens, and initiate immune response through humoral or cellular mechanisms.

Components of the humoral immune system have important roles in the inflammatory responses to foreign materials. Antibodies and complement components C3b and C3bi adhere to foreign materials, act as opsonins, and facilitate phagocytosis of the foreign materials by neutrophils and macrophages that have cell-surface receptors for C3b. Complement component C5a is a chemotactic agent for neutrophils, monocytes, and other inflammatory cells and facilitates the immigration of these cells to the implant site. The complement system is composed of classic and alternative pathways that eventuate in a common pathway to produce the membrane attack complex, which is capable of lysing microbial agents. The complement system (i.e., complement cascade) is closely controlled by protein inhibitors in the host cell membrane that may prevent damage to host cells. This inhibitory mechanism may not function when non-host cells are used in tissue-engineered devices.

T (thymus-derived) lymphocytes are significant cells in the cell-mediated adaptive immune response and their cell-adhesion molecules have a significant role in lymphocyte migration, activation, and effector function. The specific interaction of cell membrane adhesion molecules, sometimes also called ligands or antigens, with APCs produces specific types of lymphocytes with specific functions. Table 39.5 indicates cell types and function in the adaptive immune response. Obviously, the functions of these cells are more numerous than those indicated in the table, but the major function of these cells is provided to indicate similarities and differences in the interaction and responsiveness of these cells. Effector T cells (Table 39.6) are produced when their antigen-specific receptors and either the CD4 or the CD8 co-receptors bind to peptide−major histocompatibility complexes (MHCs). A second, costimulatory signal is also required, which is provided by the interaction of the CD28 receptor on the T cell and the B7.1 and B7.2 glycoproteins of the Ig superfamily present on APCs. B lymphocytes bind soluble antigens through their cell-surface Ig and thus can function as professional APCs by internalizing the soluble antigens and presenting peptide fragments of these antigens as MHC−peptide complexes. Once activated, T cells can synthesize the T-cell growth factor IL-2 and its receptor. Thus, activated T cells secrete and respond to IL-2 to promote T cell growth in an autocrine fashion.

TABLE 39.5 Cell Types and Function in Adaptive Immune System

Cell Type	Motor Function
Macrophages (antigen-presenting cell [APC])	Process and present antigen to immunocompetent T cells
	Phagocytosis
	Activated by cytokines (i.e., interferon-gamma) from other immune cells
T cells	Interact with APCs and are activated through two required cell membrane interactions
	Facilitate target cell apoptosis
	Participate in transplant rejection (type IV hypersensitivity)
B cells	Form plasma cells that secrete immunoglobulins (IgGs) (IgA and IgE)
	Participate in antigen−antibody complex mediated tissue damage (type III hypersensitivity)
Dendritic cells (APC)	Process and present antigen to immunocompetent T cells
	Use Fc receptors for IgG to trap antigen−antibody complexes
Natural killer cells (non-T, non-B lymphocytes)	Innate ability to lyse tumor, virus-infected, and other cells without previous sensitization
	Mediates T- and B-cell function by secretion of interferon-gamma

TABLE 39.6 Effector T Lymphocytes in Adaptive Immunity

T-Helper 1 Cells	CD41
	Proinflammatory
	Activation of macrophages
	Produces IL-2, IFN-gamma, IL-3, TNF-α, GM-CSF, macrophage chemotactic factor, and migration inhibitor factor
	Induces IgG2a
Th2 helper cells	CD41
	Antiinflammatory
	Activation of B cells to make antibodies
	Produces IL-4, IL-5, IL-6, IL-10, IL-3, GM-CSF, and IL-13
	Induces IgG1
Cytotoxic T cells	CD81
	Induces apoptosis of target cells
	Produces IFN-gamma, TNF-β, and TNF-α
	Releases cytotoxic proteins

GM-CSF, granulocyte macrophage—colony-stimulating factor; *IFN*, interferon; *Ig*, immunoglobulin; *IL*, interleukin; *Th*, T helper; *TNF*, tumor necrosis factor.

Cytokines are the messenger molecules of the immune system. Most cytokines have a wide spectrum of effects, reacting with many different cell types, and some are produced by several different cell types. Table 39.7 presents common categories of cytokines and lists some of their general properties. Whereas cytokines can be subdivided into functional groups, many cytokines such as IL-1, TNF-α, and IFN-gamma are pleotropic in their effects and regulate, mediate, and activate numerous responses by various cells.

Immunotoxicity is any adverse effect on the function or structure of the immune system or other systems as a result of an immune system dysfunction [93]. Adverse or immunotoxic effects occur when humoral or cellular immunity needed by the host to defend itself against infections or neoplastic disease (immunosuppression) or unnecessary tissue damage (chronic inflammation, hypersensitivity, or autoimmunity) is compromised. Potential immunological effects and responses that may be associated with one or more of these effects are presented in Table 39.8. Hypersensitivity responses are classified on the basis of the immunological mechanism that mediates the response. There are four types: I (anaphylactic), II (cytotoxic), III (immune complex), and IV (cell-mediated delayed hypersensitivity). Hypersensitivity is considered to be increased reactivity to an antigen to which a human or animal has been previously exposed, with an adverse rather than a protective effect. Hypersensitivity is a synonym for allergy. Type I (anaphylactic) reactions and type IV (cell-mediated delayed hypersensitivity) reactions are the most common [94]. Types II and III reactions are relatively rare and are less likely to occur with medical devices and biomaterials; however, with tissue-engineered devices containing potential antigens (i.e., proteins), extracellular matrix (ECM) components, and/or cells, types II and III reactions must be considered in biological response evaluations.

Type I (anaphylactic) hypersensitivity reactions are mediated by IgE antibodies, which are cytotropic and affect the immediate release of basoactive amines and other mediators from basophils and mast cells followed by the recruitment of other inflammatory cells. Type IV cell-mediated (delayed) hypersensitivity responses involve sensitized T lymphocytes that release cytokines and other mediators that lead to cellular and tissue injury. Type IV hypersensitivity (cell-mediated) reactions are initiated by specifically sensitized T lymphocytes. This reaction includes the classic delayed-type hypersensitivity reaction initiated by CD4+ T cells and direct cell cytotoxicity mediated by CD8+ T cells. The less common type II (cytotoxic) hypersensitivity involves the formation and binding of IgG and/or IgM to antigens on target cell surfaces that facilitate phagocytosis of the target cell or lysis of the target cell by activated complement components. Type II hypersensitivity (cytotoxic) is mediated by antibodies directed toward antigens present on the surface of cells or other tissue components. Three different antibody-dependent mechanisms may be involved in this type of reaction: complement-dependent reactions, antibody-dependent

TABLE 39.7 Selected Cytokines and Their Effects

Cytokine	Effect
IL-1, TNF-α, INF-gamma, and IL-6	Mediate natural immunity
IL-1, TNF-α, and IL-6	Initiate nonspecific inflammatory responses
IL-2, IL-4, IL-5, IL-12, IL-15, and TGF-β	Regulate lymphocyte growth, activation, and differentiation
IL-2 and IL-4	Promote lymphocyte growth and differentiation
IL-10 and TGF-β	Downregulate immune responses
IL-1, INF-gamma, TNF-α, and migration inhibitor factor	Activate inflammatory cells
IL-8	Produced by activated macrophages and endothelial cells
	Chemoattractant for neutrophils
Monocyte chemoattractant protein-1, macrophage inflammatory protein-α, and Regulated on Activation, Normal T Expressed and Secreted	Chemoattractant for monocytes and lymphocytes
GM-CSF and G-CSF	Stimulate hematopoiesis
IL-4 and IL-13	Promote macrophage fusion and foreign body giant cell formation

G-CSF, granulocyte—colony-stimulating factor; *GM-CSF*, granulocyte macrophage—colony-stimulating factor; *IFN*, interferon; *IL*, interleukin; *TGF*, transforming growth factor; *TNF*, tumor necrosis factor.

TABLE 39.8 Potential Immunological Effects and Responses

Effects	Responses
Hypersensitivity	Histopathological changes
Type I: anaphylactic	Humoral responses
Type II: cytotoxic	Host resistance
Type III: immune complex	Clinical symptoms
Type IV: cell-mediated (delayed)	Cellular responses
Chronic inflammation	T cells
Immunosuppression	Natural killer cells
Immunostimulation	Macrophages
Autoimmunity	Granulocytes

reactions, cell-mediated cytotoxicity, or antibody-mediated cellular dysfunction. Type III immune complex hypersensitivity is present when circulating antigen—antibody complexes activate complement whose components are chemotactic for neutrophils that release enzymes and other toxic moieties and mediators leading to cellular and tissue injury.

Immunological reactions that occur with organ transplant rejection also offer insight into potential immune responses to tissue-engineered devices. Mechanisms involved in organ transplant rejection include T cell—mediated reactions by direct and indirect pathways and antibody-mediated reactions. Immune responses may be avoided or diminished by using autologous or isogeneic cells in cell—polymer scaffold constructs. The use of allogeneic or xenogeneic cells incorporated into the device requires the prevention of immune rejection by immune suppression of the host, induction of tolerance in the host, or immunomodulation of the tissue-engineered construct. The development of tissue-engineered constructs by immunoisolation using polymer membranes and the use of non-host cells have been compromised by immune responses. In this concept, a polymer membrane is used to encapsulate non-host cells or tissues, thus separating them from the host immune system. However, antigens shed by encapsulated cells were released from the device and initiated immune responses [42,95,96].

Although exceptionally minimal and superficial in its presentation, the previously discussed humoral and cell-mediated immune responses demonstrate the possibility that any known tissue-engineered construct may undergo immunological tissue injury. To date, our understanding of immune mechanisms and their interactions with tissue-engineered constructs is markedly limited. An obvious problem is that preliminary studies are generally carried out with nonhuman tissues, and immune reactions result when tissue-engineered constructs from one species are used to test the device in another species. Ideally, tissue-engineered constructs would be prepared from cells and tissues of a given species and subsequently tested in that species. Although this approach does not guarantee that immune responses will not be present, the probability of immune responses in this type of situation is markedly decreased.

The following examples provide perspective to these issues. They further demonstrate the detailed and in-depth approach that must be taken to evaluate tissue-engineered constructs or devices and their potential adverse responses appropriately and adequately.

The inflammatory response considered to be immunotoxic is persistent chronic inflammation. With biomaterials, controlled release systems, and tissue-engineered devices, potential antigens capable of stimulating the immune response may be present and these agents may facilitate a chronic inflammatory response that is of extended duration (weeks or months). Regarding immunotoxicity, this persistent chronic inflammation is of concern because immune granuloma formation and other serious immunological reactions such as autoimmune disease may occur. Thus, in biological response evaluation, it is important to discriminate between the short-lived chronic inflammation that is a component of the normal inflammatory and healing responses versus long-term, persistent chronic inflammation that may indicate an adverse immunological response.

Immunosuppression may occur when antibody and T-cell responses (adaptive immune response) are inhibited. Potentially significant consequences of this type of response are frequent, and serious infections result from reduced host defense. Edelman and colleagues demonstrated that incorporating endothelial cells into three-dimensional collage matrices has a downregulating effect on the humeral and cellular immune response elicited by the endothelial cells [97]. The strong MHC-dominant immune response that occurs when endothelial cells are the primary component of an implant can be significantly reduced when the endothelial cells are embedded in the three-dimensional collagen matrix. Although they retain many of the characteristics of quiescent endothelial cells, the endothelial cells evoke no significant humeral or cellular immune response in immunocompetent animals and also reduce the memory response to previous free endothelial cell implants. These studies are significant and demonstrate the influence of spatial matrix formation as well as matrix composition on endothelial cell immunophenotype. Thus, modulation of the matrix structure may be helpful in designing vascular conduits for tissue-engineered devices.

Using ECM scaffolds prepared under different conditions, Badylak and colleagues determined the participation of different macrophage phenotypes in the degradation and remodeling of the ECM scaffolds, demonstrating that the properties of the matrix can control the innate and possibly acquired immune responses to ECM scaffolds [98–100].

Immunostimulation may occur when unintended or inappropriate antigen-specific or nonspecific activation of the immune system is present. From a biomaterial and controlled-release system perspective, antibody and/or cellular immune responses to a foreign protein may lead to unintended immunogenicity. Enhancement of the immune response to an antigen by a biomaterial with which it is mixed ex vivo or in situ may lead to adjuvancy, which is a form of immunostimulation. This effect must be considered when biodegradable controlled-release systems are designed and developed for use as vaccines [101–103].

Patients implanted with polyurethanes used for left ventricular assist devices (LVADs) experience B-cell hyperreactivity and allosensitization [104]. There is evidence that T lymphocytes can be activated in response to biomaterials. T lymphocytes cultured in the presence of polyurethane particles from the flexible diaphragms of LVADs resulted in intracellular calcium flux, CD40 ligand expression, and nuclear translocation of nuclear factor (NFAT) of activated T cells. NFAT translocation was reduced by a calcineurin inhibitor and CD40 ligand expression was reduced by both a calcineurin inhibitor and CD25 blockade indicating IL-2–dependent activation pathways [104,105]. In response to polyurethane particles, T lymphocytes exhibited classic activation indicators: calcium flux, translocation of transcription factors, upregulation of activation cell surface markers, and proliferation.

Differences in human leukocyte antigen (HLA) gene inheritance can result in MHC diversity. MHC loci are among the most genetically variable loci in humans. The MHC class II proteins (DP, DQ, and DR) are found on APCs. Diversity in MHCII proteins results in individual variability in antigen presentation and thus immune responses. Because of this diversity, individuals mount immune responses to different epitopes of pathogens. LVAD recipients who are predisposed to develop B-cell hyperreactivity have HLA-DR3 expression, which indicates

that lymphocyte responses to biomaterials are variable and depend on the individual's genetic profile [106]. It is possible that only individuals with certain MHCII receptors can interact with biomaterials in a mechanism that results in a lymphocyte response.

Autoimmunity is the immune response to the body's own constituents, which are considered in this response to be autoantigens. An autoimmune response, indicated by the presence of autoantibodies or T lymphocytes that are reactive with host tissue or cellular antigens, may (or may not) result in autoimmune disease with chronic, debilitating, and sometimes life-threatening tissue and organ injury.

Representative tests to evaluate for immune responses are given in Table 39.9. The table is not all-inclusive; other tests may be applicable. The examples presented represent only the large number of tests that are currently available [87,88,92]. The table is informative but incomplete, because in the future, direct and indirect markers of immune response may be validated and their predictive value documented, thus providing new tests for immunotoxicity. Direct measures of immune system activity by functional assays are the most important types of tests for immunotoxicity. Functional assays are generally more important than are tests for soluble mediators, which are more important than phenotyping. Signs of illness may be important in in vivo experiments, but symptoms may also have a significant role in studies of immune function in clinical trials and postmarket studies.

As with any type of test for biological response evaluation, immunotoxicity tests should be valid; they have been shown to provide accurate, reproducible results that indicate the effect being studied and are useful in a statistical analysis. This implies that appropriate control groups are also included in the study design.

Immunogenicity involving a specific immune response to a biomaterial is an important consideration because it may lead to serious adverse effects. For example, a foreign, nonhuman protein may induce IgE antibodies that cause an anaphylactic (type I) hypersensitivity reaction. An example of this type of response is latex protein found in latex gloves. Low—molecular weight compounds such as chemical accelerators used to manufacture latex gloves may also induce a T cell—mediated (type IV) reaction, resulting in contact dermatitis. Tests for type I (e.g., antigen-specific IgE) and type IV (e.g., guinea pig) maximization hypersensitivity should be considered for materials with the potential to cause these allergic reactions. In addition to hypersensitivity reactions, a device may elicit autoimmune responses (i.e., antibodies or T cells) that react with the body's own constituents. An autoimmune response may lead to the pathological consequences of an autoimmune disease. For example, a foreign protein may induce IgG or IgM

TABLE 39.9 Representative Tests for Evaluation of Immune Responses

Functional Assays	**Soluble Mediators**
Skin testing	Antibodies
Immunoassays (e.g., enzyme-linked immunosorbent assays)	Complement
Lymphocyte proliferation	Immune complexes
Plaque-forming cells	Cytokine patterns (T-cell subsets)
Local lymph node assay	Cytokines (IL-1, IL-1ra, TNF-α, IL-6, TGF-β, IL-4, and IL-13)
Mixed lymphocyte reaction	Chemokines
Tumor cytotoxicity	Basoactive amines
Antigen presentation	**Signs of illness**
Phagocytosis	
Degranulation	Allergy
Resistance to bacteria, viruses, and tumors	Skin rash
Phenotyping	Urticaria
	Edema
Cell-surface markers	Lymphadenopathy
Major histocompatibility complex markers	

IL, interleukin; *TNF*, tumor necrosis factor; *TGF*, transforming growth factor.

antibodies that cross-react with a human protein and cause tissue damage by activating the complement system. In a similar fashion, a biomaterial or controlled-release system that has a gel or oil constituent may act as an adjuvant, leading to the induction of an autoimmune response. Even if an autoimmune response (autoantibodies and/or autoreactive T lymphocytes) is suggested in preclinical testing, it is difficult to obtain convincing evidence that a biomaterial or controlled-release system causes autoimmune disease in animals. Therefore, routine testing for the induction of autoimmune disease in animal models is not recommended.

Babensee and coworkers tested the hypothesis that by promoting an inflammatory response, the biomaterial component of a medical device can recruit APCs (e.g., macrophages and dendritic cells) and induce their activation, thus acting as an adjuvant in the immune response to foreign antigens originating from the histological component of the device [107–109]. Using polystyrene and polylactic-glycolic acid microparticles and polylactic-glycolic scaffolds together with their model antigen, ovalbumin, in a mouse model for 18 weeks, the researchers demonstrated that a persistent humoral immune response that was Th2, T-cell dependent, as determined by IgG1, was present. These findings indicated that activation of CD41 T cells and the proliferation and isotype switching of B cells had occurred. A Th1 immune response characterized by the presence of IgG2a was not identified. Moreover, the humoral immune responses for all three types of microparticles were similar, indicating that the production of antigen-specific antibodies was not material chemistry–dependent in this model. Babensee et al. suggested that the presence of the biomaterial functions as an adjuvant for initiation and promotion of the immune response and augments the phagocytosis of the antigen with expression of MHCII and costimulatory molecules on APCs with the presentation of antigen to CD41 T cells.

Babensee and coworkers identified differential levels of dendritic cell maturation on different biomaterials used in combination products [110–114]. The effect of biomaterials on dendritic cell maturation, and the associated adjuvant effect, are novel biocompatibility selection and design criteria for biomaterials to be used in combination products in which immune consequences are potential complications or outcomes.

Badylak and colleagues carried out extensive studies on the use of xenogeneic ECM as a scaffold for tissue reconstruction [115–117]. Use of the small intestinal submucosa (SIS) ECM in animals indicated a restricted Th2-type immune response. The presence of natural antibodies to the terminal galactose-α1,3-galactose (α-gal) epitope is considered to be a major barrier to xenotransplantation in humans. Cell membranes of all animals except humans express this epitope, and naturally occurring antibodies mediate hyperacute or delayed rejection of transplanted organs through complement fixation or antibody dependence cell-mediated cytotoxicity. Whereas ECM derived from porcine tissues, SIS, contains small amounts of the galactose epitope, it appears that the quantity or distribution of this epitope and/or the subtype of Ig response to the epitope is such that complement activation does not occur [118]. In addition, the resorbable characteristics of this nonchemically cross-linked ECM scaffold demonstrate constructive tissue remodeling and deposition of new matrix whereas chemically cross-linked ECM leads to active inflammation and eventually scar formation.

The role of Th1 and Th2 lymphocytes in cell-mediated immune responses to xenografts has been examined. Activation of the Th1 pathway leads to macrophage activation, stimulation of complement fixing antibody isotypes, and differentiation of CD8+ cells to a cytotoxic type phenotype that is associated with both allogeneic and xenogeneic transplant rejection. The Th2 lymphocyte response does not activate macrophages and leads to production of noncomplement fixing antibody isotypes; it is usually associated with transplant acceptance.

The use of appropriate animal models is an important consideration in the safety evaluation of controlled-release systems that may contain potential immunoreactive materials [91,119,120]. A study involving the in vivo evaluation of recombinant human growth hormone in poly(lactic-*co*-glycolic acid) (PLGA) microspheres demonstrated the appropriate use of various animal models to evaluate biological responses and the potential for immunotoxicity. Using biodegradable PLGA microspheres containing recombinant human growth hormone (rhGH), Cleland et al. used rhesus monkeys, transgenic mice expression rhGH, and normal control (Balb/C) mice in their in vivo studies [112]. Rhesus monkeys were used for serum assays in the pharmacokinetic study of rhGH release as well as tissue responses to the injected microcapsule formulation. Placebo injection sites were also employed, and a comparison of the injection sites from rhGH PLGA microspheres and placebo PLGA microspheres demonstrated a normal inflammatory and wound healing response with a normal focal foreign body reaction. To examine the tissue response further, transgenic mice were used to assess the immunogenicity of the rhGH PLGA formulation. Transgenic mice expressing a heterologous protein were previously used to assess the immunogenicity of sequence or structural mutant proteins [121,122]. With the transgenic animals, no detectable antibody response to rhGH was found. In contrast, the Balb/C control mice had a rapid onset of high titer antibody response to the rhGH PLGA formulation. This study pointed out the appropriate use of animal models not only to evaluate biological responses but also for one type of immunotoxicity (immunogenicity) of controlled-release systems.

The focus in tissue engineering traditionally has been on modulating the fate of transplanted host cell populations that participate directly in reconstructing tissues. However, new materials for tissue engineering are being considered that give greater control over the inflammatory and immune responses [123]. Biomimetic strategies based on viruses and bacteria are being used to develop immune evasive biomaterials [124]. Materials are being investigated that can promote tolerance to specific antigens and cells by directly signaling APCs such as dendritic cells, or by releasing growth factors or cytokines that promote tolerance. On the other hand, materials might promote a destructive immune response by directly providing immunity-promoting signals or releasing insoluble factors. This approach could be used to combat infections and cancer [125].

CONCLUSION

Tissue-engineered devices are biological−biomaterial combinations in which some component of tissue has been combined with a biomaterial to create a device for the restoration or modification of tissue or organ function. The biocompatibility and bioresponse require the ultimate achievement of four significant goals if these devices are to function adequately and appropriately in the host environment. These goals are (1) restoration of the target tissue with its appropriate function and cellular phenotypic expression; (2) inhibition of the macrophage and FBGC foreign body response that may degrade or adversely modify device function; (3) inhibition of scar and fibrous capsule formation that may be deleterious to the function of the device; and (4) inhibition of immune responses that may inhibit the proposed function of the device and ultimately lead to the destruction of the tissue component of the tissue-engineered device. This chapter has presented a brief and limited overview of mechanisms and biological responses that determine biocompatibility: inflammation, wound healing, and immunotoxicity. Given the unique nature of the combination of tissue components and biomaterials in tissue-engineered devices, coupled with the species differences in biological responses, a significant future challenge in developing tissue-engineered devices is to construct and use a unique set of tests that will ensure that these four goals are achieved for the lifetime of the device in its in vivo environment in humans.

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Further Reading

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CHAPTER

40

Hybrid Composite Biomaterials

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INTRODUCTION

Damage and degeneration of tissues caused by disease, injury, and trauma to the human body necessitate treatments including transplanting tissue from one site to another in the same patient (an autograft) or from one individual to another (a transplant or allograft). All techniques have the serious constraints of being expensive and painful and having the risks for rejection by the patient's immune system and the possibility of introducing infection or disease from the donor to the patient. Therefore, the approach of tissue engineering to repair defects in tissues is perceived as a smart strategies because the repair process proceeds with the patient's own tissue. The field of tissue engineering is highly multidisciplinary; it has the aim of developing biological substitutes to regenerate damaged tissues and to restore, maintain, or improve tissue function [1,2]. The tissue engineering triad is mainly composed of three main key components of engineered tissues: cells, scaffolds, and the growth-stimulating signal. Typically, the three-dimensional (3D) scaffolds are made from natural or synthetic biomaterials in various formats, serving as a template to provide an appropriate environment for tissue regeneration. These tissue engineered scaffolds are mainly seeded with cells and growth factors or subjected to biophysical stimuli in a bioreactor (a device that applies different types of mechanical or chemical stimuli to cells) [3]. These cell-seeded scaffolds are then either cultured in vitro to produce tissues followed by implantation into an injured site or directly implanted into the injured target site using the body's own systems in which tissues regeneration is induced in vivo [4]. In this chapter, we outline various materials used for scaffolding, the functions of scaffolds, approaches to scaffolding, and tissue-specific considerations for scaffolding, using bone as an example.

Materials that are implanted to repair, replace, or augment existing tissues in the body are generally known as biomaterials. In the wider context covered in this chapter, biomaterials include all materials formed in biological systems, e.g., the specific products of biomineralization. The development of biomaterials, both as products and in understanding their in vivo behavior, has been driven largely by the desire to assist in caring for patients. The material-forming processes occurring in living organisms require much milder reaction conditions than are currently used in the laboratory, such that a new area of materials chemistry, "biomimetics," has been established in which scientists are taking ideas from biology to generate "softer" routes to useful materials.

Biomaterials present in nature provide the necessary structure and architectures of all animal and plant species on earth and function to maintain the structure of organs as well as the organism itself. In nature, materials that are used are polymers such as polysaccharides and proteins and a relatively small number of simple insoluble oxides and salts. These can be put together in a wide range of combinations to produce materials that are soft, hard, flexible, elastic, etc. In contrast, the range of available materials for biomedical applications is vast and includes metals, polymers, ceramics, and their composites. In designing medical devices, materials are chosen to suit their intended use and the proposed implantation area. The materials that are used must have properties that are compatible with the location in which they are placed. Properties such as the tensile strength, toughness, elasticity, and hardness have to be considered, and other factors such as material transparency may have to be thought about if, for instance, the device is to be used within the eye.

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Although technologists have a wider array of materials at their disposal, it is not a simple matter to come up with a material or series of materials that fulfil all of the criteria required for successful implantation or biomedical use. In the wider context covered in this chapter, biomaterials include all materials formed in biological systems, e.g., the specific products of biomineralization. The development of biomaterials, both as products and in understanding their in vivo behavior, has been driven largely by the desire to assist in caring for patients. The material-forming processes occurring in living organisms require much milder reaction conditions than are currently used in the laboratory, such that a new area of materials chemistry, "biomimetics," has been established in which scientists are taking ideas from biology to generate "softer" routes to useful materials.

FUNDAMENTALS OF BONE DEVELOPMENT AND DEFECTS

Bone is a calcium reservoir in the body in the form of hydroxyapatite (HA) (85%), calcium carbonate (10%), calcium fluoride (~3%), and magnesium fluoride (~3%). There are two main structural types in the bone: cortical bone forms a dense outer shell and cancellous or trabecular bone forms the porous inner core. Cortical bone is highly dense structure consisting of hierarchical structures, each within a different size scale; it provides torsion, bending resistance, and compressive strength. On the other hand, cancellous bone is highly porous and possesses an interconnected trabeculae network filled with marrow, a hierarchical structure ranging from solid material to trabeculae, lamellae, and a collagen–mineral composite. The trabeculae have a large surface area for the diffusion of nutrients and the circulation of growth factors, which allows cancellous bone tissue to be metabolically active and which can be remodeled more frequently than that of cortical bone.

Bone formation occurs via two distinctive pathways: intramembraneous and endochondral. First, mesenchymal cellular condensation occurs and acts as a template for subsequent bone formation. Intramembraneous bone formation involves the differentiation of mesenchymal progenitor cells directly into osteoblasts; it further develops into parts of the mandible, clavicle, and many cranial bones. Most of the bones in the human body, including all of the long bones and vertebrae, were formed through endochondral bone formation, in which mesenchymal progenitor cells differentiate into chondrocytes, which are responsible for cartilaginous template formation, and then are further mineralized and reinstated by bone.

Upon fracture, the bone becomes repaired by recapitulating several events of endochondral and intramembraneous bone formation and heals with no scar tissue formation. Usually, the formation of hematoma involves an inflammatory response and many signaling molecules specific for the regulation of new bone formation (i.e., tumor necrosis factor-α, interleukins, fibroblast growth factors, platelet-derived growth factor, vascular endothelial growth factor [VEGF], bone morphogenetic proteins [BMPs], etc.). The formation of intramembranous bone occurs immediately at the cortex and periosteum. Then the fracture becomes stabilized by the external soft tissues via callus formation; subsequently, chondrocyte proliferation takes place. Then the ingrowing blood vessels carry chondroclasts, which reabsorb calcified cartilage and osteoblastic progenitors and initiate new bone formation. The mechanical continuity of the cortex is achieved via subsequent remodeling of the newly formed bone. In case of damage or disease requiring bone tissue regeneration, the formation of hematoma and an early inflammatory response occurs, which facilitates host cell recruitment and the release of critical signaling molecules. Thus, smart scaffolds mimicking the properties of normal bone tissue development are important to efficient bone tissue engineering. Fundamental developmental biology principles that should be considered for bone tissue engineering are: (1) the use of pluripotent or multipotent stem cells; (2) the establishment of required growth factors, involved genes, and signal transduction cascades facilitating bone formation; (3) an understanding of the bone formation process and normal tissue healing; (4) an understanding of complex interactions between mesenchyme and epithelium, and mesenchyme-encoding, tissue-specific patterns; (5) an understanding of the criticality of the tissue microenvironment's physical characteristics (mechanotherapy, etc.); and (6) neovascularization and angiogenesis of the newly formed and developed bone tissue.

FUNCTIONS OF SCAFFOLDING AND EXTRACELLULAR MATRIX

Various tissue engineered constructs or scaffolds have been extensively fabricated from numerous materials and a plethora of manufacturing approaches for the neoregeneration of different tissues and organs in the body. When designing an ideal scaffold for tissue engineering, a number of important features and functions must be considered regardless of the type of tissue:

Architecture: Scaffolds should possess an interconnected pore structure and high porosity and void volume for proper vascularization, the formation of neotissue, and remodeling, which facilitates cell penetration and host tissue integration upon implantation. The biomaterial must provide enough interconnected porous structure for efficient nutrient and metabolite diffusion to cells within the scaffolds and to the extracellular matrix (ECM) formed by these cells without significantly compromising mechanical scaffold stability. Moreover, the scaffold should have enough mean pore size for cell migration within the structure where they eventually bind to the ligands present in scaffold materials, but small enough to establish a sufficiently high specific surface leading to a minimal ligand density to allow efficient binding of a critical number of cells to the scaffold [5]. Thus, for any scaffold, a critical range of pore sizes exists that may vary depending on the cell type used and the tissue being engineered [6]. Furthermore, the materials must be degradable upon implantation at a rate corresponding to the newly formed matrix by the developing tissue. The issue of core degradation, which arises from the lack of vascularization and waste removal from scaffolds, is one of the major concerns in the tissue engineering field [7,8].

Cytocompatibility: An important criterion for any tissue engineered scaffold is that it must be biocompatible: seeded cells must adhere, function normally, migrate onto or through the scaffold surface, and start proliferating during both in vitro culture and in vivo implantation before laying down new matrix. After implantation, the scaffold must be compatible with the cellular components and endogenous cells in host tissue and thus must elicit a negligible immune reaction or inflammatory response to avoid reduced healing or rejection by the body.

Bioactivity: Scaffolds must interact actively with the cellular components of the engineered tissues to regulate their activities. The construct material must be composed of biological cues such as cell-adhesive ligands to enhance attachment or physical cues such as topography to influence cell morphology and alignment. It can also act as a delivery vector for exogenous growth-stimulating signals to speed up regeneration such as growth factors. For example, hydrogels synthesized by covalent or ionic cross-linking can encapsulate proteins and perform a responsive release by hydrogels swelling [9].

Biodegradability: Scaffolds implanted for tissue engineering purposes were intended to be eventually replaced by the body's own cells. Thus, the scaffold material must be biodegradable, facilitating cells to generate their own ECM and produce nontoxic by-products followed by their removal from the body with no interference from other organs. Moreover, an inflammatory response with the infusion of controlled cells such as macrophages is essential for the execution of materials degradation with the formation of tissues or cells.

Mechanical characteristics: From a practical point of view, a scaffold should be strong enough to allow surgical handling during implantation. Moreover, its mechanical characteristics must be similar to the implantation anatomical site, providing mechanical and shape firmness to the tissue defect. Especially for orthopedic and cardiovascular applications, it is critical to design a scaffold with sufficient mechanical integrity mimicking host bone or cardiac tissue integrity. This becomes more challenging with age-dependent defects or fractures; for example, generally, fractures heal to an acceptable weight-bearing point at about 6 weeks in young individuals, but the repair rate slows down in elderly people. With the evolution of tissue engineering and regenerative medicine, the focus has been mostly diverted to manufacturing scaffolds with good mechanical properties and reduced porosity, which have been shown to have potential in vitro; however, these were unsuccessful when implanted in vivo owing to their insufficient capacity for vascularization. Thus, a well-established balance between the scaffold mechanical features and porous architecture allowing cell infiltration and vascularization is a vital factor for the success of any tissue engineered scaffold [4].

Except for blood cells, normal cells in human tissues are anchorage-dependent and located in a solid matrix called the ECM. There are various types of ECM in human tissues consisting of multiple components and a tissue-specific composition. Generally, functions of the ECM in tissues can be divided into five categories: (1) it provides a physical environment and structural support to cells in specific tissues to attach, grow, migrate, and respond to signals; (2) it provides mechanical and structural strength to tissues including elasticity and rigidity; (3) it actively provides bioactive cues to native cells to regulate their activities; (4) it behaves as a reservoir for growth factors and potentiates their bioactivities; and (5) it provides a degradable physical platform, allowing vascularization and remodeling in response to developmental, physiological, and pathological challenges during tissue dynamic processes such as morphogenesis, homeostasis, and wound healing, respectively. Thus, it is important for any ideal scaffold material to mimic the dynamic nature of the ECM in native tissues, at least partially [10].

SCAFFOLDING APPROACHES IN BONE TISSUE ENGINEERING

In this section, we discuss various approaches employed in bone tissue engineering from a materials point of view; further details are elaborated on in later sections.

Osteoinductive Materials

Osteoinductive refers to materials having the ability to induce the formation of ectopic bone by summoning the surrounding environment to form neobone [11−13]; thus, it holds immense potential for bone tissue regeneration. Materials including natural and synthetic ceramics (for example, HA, alumina ceramic, and porous Bioglass) [14] and several calcium phosphate compositions and their composites (for example, HA−poly[lactic-*co*-glycolic acid] [PLGA]) have been reported to be efficient osteoinductive materials in various bone tissue engineering studies. In particular, calcium phosphate−based designs (i.e., cements [15,16], coatings [17−19], sintered ceramics, and coral-derived ceramic [20,21]) have illustrated osteoinduction in several animal models. Polymer-ceramic blends (i.e., HA−PLGA) have displayed osteoinductive properties and induced ectopic bone formation [22,23]. Other than the material's chemical composition, several other factors have a critical role in determining osteoinduction, including porosity, surface properties, and nanotopography and microtopography. To explain osteoinductivity, two main hypotheses were proposed. The first is based on biomaterial surface properties that absorb and present osteoinductive factors to the surrounding cells. The second hypothesis is that calcium phosphate−based materials release calcium and phosphate ions, which influence stem cell differentiation into bone cells [13].

Immunomodulatory Materials

These systems have the ability to manipulate or modulate the immune system in favorably so as to enhance tissue regeneration. In general, the host's immune system reacts to an external implant by initiating an acute reaction to the surgical injury and recognize the alien material innately; this followed by adaptive immunity-mediated chronic inflammation in response to the recognition of specific antigens. Therefore, myriads of approaches were proposed in immune-bioengineering, highlighting the relevance of rational control over host inflammation that suggest cell-specific responses, accelerated wound healing, and bone tissue regeneration (enhanced activity of bone-forming cells and decreased activity of natural killer (NK) cells and T and B cell−mediated immunity). This includes the appropriate selection of materials, material surface functionalization and modulating agents (i.e., surface treatments and topography) and the inclusion of artificial ECM and bioactive agents. One approach is to develop biomaterials mimicking ECM composition and structure. Smith et al. showed polydioxanone and collagen−elastin composites with immune-modulating features by decreasing NK cell activity and T- and B-cell proliferation [24].

Material microscale and nanoscale architecture and its surface topography have a key role in modulating the immune system and host acute immune response upon scaffold implantation. Thus, the biomaterial surface should be functionalized to limit macrophage adhesion and activation, shielding them from protein absorption via polymer coating, delivering bioactive molecules (i.e., growth factors, antiinflammatory drugs), and thereby facilitating their fusion into foreign body giant cells. Some studies evaluated the pharmacologic modification effects of inflammatory response upon in vivo bone regeneration including cytokine-specific agents, corticosteroids, prostaglandins, nonsteroidal antiinflammatory drugs, and selective prostaglandin agonists [25]. A few studies have shown the enhanced strength of healed bone, vascularization at the fracture site, or accelerated fracture repair and regeneration with the controlled release of TP508 from various biodegradable scaffolds (PLGA microspheres and poly[propylene fumarate]) [26,27]. Although these results are interesting and promising, further detailed and advanced studies are essential that integrate inflammatory modulation strategies into tissue engineering to enhance tissue regeneration.

Hybrid Materials

Any biomaterial chosen for tissue engineering scaffolding needs to possess certain biological and physiochemical characteristics specific for the target tissue. However, it is difficult for any material to meet all of these expectations by itself; thus, hybrid materials have been made by combining two or more materials complementing each other in terms of the required features. Various optimal and advanced hybrids were developed (i.e., copolymers, polymer−polymer blends, polymer−ceramic composites) These are described in detail in later sections.

Hydrogels

Hydrogels, which have the ability to mimic ECM topography and deliver bioactive substances to promote tissue regeneration owing to their physical characteristics and biocompatibility, have become popular in tissue engineering and regenerative medicine. Examples includes both nature-derived (collagen, silk, and gelatin) and synthetic biomaterials (poly[vinyl alcohol] and poly[ethylene glycol]). Self-assembling peptides have attracted the interest of

researchers for scaffolding applications because these systems are nonimmunogenic, biocompatible, and biodegradable, and can be readily manufactured chemically and biologically to serve as a starting scaffolding matrix [28,29]. For instance, self-assembling RAD16-I (i.e., PuraMatrix, Cambridge, MA) can form an injectable nanofiber network or hydrogel upon implantation [30–32].

SCAFFOLDING MATERIALS

Natural Polymers

The major goal of bone tissue engineering is to develop bioconstructs that substitute for the functionality of damaged natural bone structures as much as possible if critical-sized defects occur. Scaffolds that mimic the structure and composition of bone tissue and cells have a pivotal role in bone tissue engineering. Natural polymers have attractive properties for the construction of 3D scaffolds, such as biocompatibility and biodegradability. The porosity, charge, and mechanical strength can be controlled by changing polymer concentrations or polymerization conditions, or by introducing various functional groups. Bioactivity can also be tuned by adding chemicals, proteins, peptides, and cells. The most commonly studied natural polymers for the purpose of bone engineering are collagen/gelatin, chitosan (CHI), silk, alginate, hyaluronic acid (HAc), and peptides [33]. Here we discuss studies in which these natural polymers were studied as 3D scaffolds for bone regeneration and were modified in different manners to improve their osteogenic capabilities.

Silk

Silk fibroin (SF) is a fibrous protein that is produced mainly by silkworms and spiders. Its unique mechanical properties and fine-tunable biodegradation rate, and the ability to support the differentiation of mesenchymal stem cells along the osteogenic lineage have made SF a favorable scaffold material for bone tissue engineering. SF can be processed into various scaffold forms that can be combined synergistically with other biomaterials to form composites and chemically modified, which provides an impressive toolbox and allows SF scaffolds to be tailored to specific applications [34]. Silk is composed of two major proteins: SF (fibrous protein) and sericin (globular protein). SF is a protein isolated from different animals in the form of an aqueous protein solution. The ability to produce silk has evolved multiple times among insects such as *Bombyx mori*, spiders, mites, and beetles with diverse functions [35].

In one study, calcium phosphate (CaP)/silk powders were incorporated into silk scaffolds to improve the porous structure and distribution of CaP powders in the composite scaffolds [36,37]. The scaffolds tested contained pure silk, or silk with 5% or 10% CaP. The pure silk and silk composite scaffolds were prepared using a freeze-drying method. The addition of CaP did not affect the compressive strength of the material (all tested were ~70 KPa) or the compressive modulus (~250 MPa for all materials tested). All scaffolds tested supported bone marrow stromal cell (BMSC) proliferation to comparable levels. However, alkaline phosphatase (ALP) activity was significantly higher in CaP–silk scaffolds at days 7 and 14. The expression of the osteogenic markers ALP, type 1 collagen, and osteocalcin (OCN) was also significantly increased in CaP–scaffolds at day 7 and 14 compared with pure silk scaffolds. In vivo bone formation was assessed in a calvarial defect model by microcomputed tomography (μCT). Groups studied were silk scaffold, CaP–scaffold, silk scaffold plus mesenchymal stem cells (MSCs), and CaP–scaffold plus MSCs. Defect areas were collected after 4 weeks. When bone formation was measured, it was clear that the addition of CaP into the scaffold significantly increased bone volume in the area. These results suggest that the presence of CaP in the scaffold was sufficient to enhance osteogenesis, because it did had no effect on the scaffold's mechanical properties. Decellularized bovine trabecular bone was used for comparison. It was found that the values of these parameters approached those for bovine trabecular bone in scaffolds with 3.1% and 4.6% HA. Calcium content was studied in all scaffolds at up to 10 weeks; there was increase over time but no differences between groups. These studies demonstrate a system in which, although there was no improvement in other work, the relationship between scaffold degradability and osteogenesis was examined [38]. 3D porous SF scaffolds were prepared with two different degradation rates. The water-based scaffold was synthesized as the rapidly degrading scaffold (control) and a slower-degrading scaffold was obtained by adding hexafluoroisopropanol (HFIP) to induce insolubility in aqueous media (HFIP scaffold). Degradation studies were performed; the control scaffold lost more than 90% of its mass at day 7 and the HFIP scaffold's mass remained nearly the same at 7 days. Various biochemical assays were performed on both scaffolds after seeding with MSCs and culturing for 16 or 56 days. At 56 days, the DNA content in the control scaffolds was around sixfold lower than for HFIP scaffolds.

There was no significant difference in ALP activity between the two scaffolds. However, calcium content/DNA and total collagen/DNA showed a dramatic increase in the rapidly degrading scaffold compared with the slowly degrading scaffold. There was no significant difference in the expression of osteogenesis-related genes ALP, bone sialoprotein (BSP), Col1a1, or osteopontin (OPN) between control and HFIP scaffolds. However, at day 56, hypoxia-inducible factor-1 (HIF-1) expression was significantly higher in the control than the HFIP scaffold. Studies in transgenic mice have shown that HIF-1 is a critical component of bone regeneration.

Another study was conducted to evaluate the ability of fibroin scaffolds combined with human stem cells, such as human dental pulp stem cells (hDPSCs) and human amniotic fluid stem cells (hAFSCs), to repair critical-size cranial bone defects [39]. These scaffolds had 85% porosity and pore diameters ranging from 10 to 250 μM, and a compressive modulus of 25.69 ± 1.61 kPa. hDPSCs and hAFSCs were seeded onto the scaffolds and grown in osteogenic media for 10 days before implantation into the cranial critical-size defect. Groups tested consisted of scaffolds without cells, scaffold plus hDPSCs, scaffold plus hAFSCs, and empty defect. Thirty days after surgery, radiograph images showed that scaffolds without cells were able to repair the area slightly, but this effect was more pronounced when either kind of stem cell was present.

Hematoxylin–eosin staining showed that vascularization was present in all scaffolds tested; however, hASFCs seemed to have a greater potential for bone regeneration because it was the only scaffold tested that showed bone in scaffold areas distant to the dura matter. Another study reported the effects of different concentrations of SF protein on a 3D scaffold pore microstructure and its effects on bone formation when cultured with BMSCs transfected with BMP-7 [36,37]. At 1 wt% silk protein, scaffolds had a porosity of 94% and a pore size ranging from 250 to 300 μM. At 2 wt% protein, porosity was 87% and the pore size was 200–250 μM. At 3.5 wt% silk protein, scaffolds had a porosity of 80% and a pore size ranging from 150 to 200 μM. At 2 wt% protein, porosity was 71% and pore size was from 80 to 150 μM. MSCs proliferated on all scaffolds, but at day 14 there was a significant decrease in the 5% scaffold. ALP activity was shown to increase in all groups, but there was significantly higher expression in the 3.5% scaffold. After 2 weeks in osteogenic medium, expression of osteogenic markers in transfected and untransfected MSCs in the various scaffolds was analyzed by reverse transcriptase–polymerase chain reaction (RT-PCR). ALP, Col1, and OCN had similar levels of expression in all scaffolds tested when they were transfected with BMP-7, except in the 5% scaffold, where there was a significant decrease in marker expression. This study indicated that decreased scaffold porosity is detrimental to the promotion of bone formation and that the presence of BMP-7 greatly enhances cells' ability to express osteogenic phenotypes.

Collagen

The ECM provides physical support to tissues by occupying the intercellular space, acting not only as benign native scaffolding for arranging cells within connective tissues but also as a dynamic, mobile, and flexible substance defining cellular behaviors and tissue function [40]. For most soft and hard connective tissues (bone, cartilage, tendon, cornea, blood vessels, and skin), collagen fibrils and their networks function as an ECM, the highly organized 3D architecture surrounding various cells. Collagen has a dominant role in maintaining the biologic structural integrity of ECM and is highly dynamic, undergoing constant remodeling for proper physiologic functions [40]. As a primary component of bone, collagen (and gelatin) is an idyllic candidate for the design of 3D scaffolds [41]. It is inherently biocompatible and biodegradable and stimulates the proliferation and differentiation of cells as an ECM. However, it has poor mechanical properties. In many studies, collagen was used as a base for 3D scaffolds and modified by adding polymers and other biomolecules to improve osteoinductivity. In one [42], the authors combined the mechanical properties of a 3D macrochanneled poly-ε-caprolactone (PCL) scaffold, fabricated by robotic dispensing, with the bioactive properties of collagen. An MSC-loaded collagen hydrogel collagen was inserted onto the macrochanneled scaffold. In addition, they studied the effect of growing these cell-seeded scaffolds in a perfusion bioreactor to test how osteogenesis was affected by the continuous supply of fresh media and shear stress. The collagen–PCL scaffolds increased cell proliferation when grown in the perfusion chamber compared with cells grown under static conditions. The activity of ALP, an early osteogenic marker, and osteogenic genes OPN, OCN, and BSP were significantly upregulated at 14 days in cells grown in the perfusion chamber. Bone marrow MSCs (BM-MSC) have shown great potential for tissue engineering purposes as they are relatively easy to isolate, retain their multipotency even after several passages, and can be induced to differentiate into bone. However, there is a low amount that can be harvested from bone marrow and they have limited proliferation and high senescence [43]. It has been reported that MSCs can be isolated from the Wharton jelly found in the umbilical cord (UC-MSC), and they are more abundant, more easily expanded, and more resistant to cryogenic storage [44]. The adhesion, migration into scaffold, growth, spreading, osteogenic differentiation, ECM degradation, and synthesis of BM-MSCs versus UC-MSCs were compared. The tested collagen scaffold (CS) showed excellent cytocompatibility

with both cell types and could maintain high proliferation and viability. When stimulated with osteogenic induction medium, both cell types showed comparable osteogenic gene expression, migration and scaffold colonization, and the ability to contract the CS. UC-MSCs were much more capable of producing an ECM. They demonstrated at least 10-fold higher expression of the ECM marker genes collagen I, collagen III, collagen IV, and laminin than did BM-MSCs. Because the production of ECM is an integral component of bone regeneration, UC-MSCs could have a significant impact on bone tissue engineering; its osteogenic potential should be investigated in additional scaffold compositions.

In another study, the hydraulic permeability (k) of a collagen-based scaffold was manipulated to improve mechanical properties, cell−scaffold interactions, oxygen flow, and nutrient diffusion [45]. Permeability is the ability of porous structures to transfer fluids through their interstices under applied pressure. This can be controlled through pore size, number, orientation, distribution, and interconnectivity [46]. Collagen scaffolds were prepared and exposed to plastic compression using different static stresses to control permeability. Results showed that increasing compression reduced k. It was also found that a decrease in k correlated with an increase in the modulus and permeability of collagen gels. The authors then tested the effect of k on MSC proliferation, differentiation, and mineralization. Compared with noncompressed gels, compressed ones showed higher proliferation, ALP staining, and mineralization, but no significant difference was found between the different compressed gels. These findings suggest that decreasing k provides a good matrix for cell proliferation and osteodifferentiation, but the influence of k on osteoinduction and osteoconduction has not been fully defined.

BMP-4 was spatially immobilized in a collagen−PLGA hybrid scaffold and has been shown to induce the osteogenic differentiation of osteoblasts to promote bone formation. MSCs were loaded into collagen−PLGA scaffolds with or without BMP-4 and cultured in osteoinductive media before implantation into the dorsa of athymic nude mice. Type 1 collagen, OPN, and OCN showed a significant increase in expression compared with the control. However, there was no difference in ALP expression. This may have been because ALP expression increased before the analysis of the removed implants. In another study, a collagen-based silicified matrix was loaded with stromal cell−derived factor-1 (SDF-1) [47], which is a chemokine-receptor ligand that is involved in immobilizing and homing stem cells to injured tissues. The silicified collagen scaffold (SCS) was first compared with CS in terms of its mechanical properties. The tangent modulus values (KPa) from 0%−5% strain were 0.80 ± 0.21 for CS and 599.8 ± 166.0 for SCS. The modulus of resilience values (KPa) were 0.18 ± 0.06 for CS and 165.3 ± 4.0 for SCS. In vitro analyses showed that there was no difference in cell viability when MSCs or endothelial progenitor cells (EPCs) were in contact with CS or SCS. The formation of extracellular bone nodules in differentiated MSCs was significantly higher in SCS than in CS, and the formation of capillary-like tubes by EPCs was much higher in SCS than in CS. These results indicate that the presence of silica in collagen hydrogels increases its osteogenic and angiogenic potential. The release kinetics of the SDF-1 from SCS hydrogels was analyzed and it was found that at high concentrations, up to 80% release could be obtained at 30 days. In vitro cell homing experiments were conducted with MSCs and EPCs using a transwell migration assay, employing SDF-1 concentrations that corresponded to those used in the release assay. Both MSCs and EPCs showed higher migration with higher SDF-1 concentrations. For in vivo experiments, the ability to promote osteogenesis was compared between SCS loaded with MSCs (cell-seeding approach) and cell-free SDF-1 containing SCS (cell-homing approach). Scaffolds were implanted into subcutaneous pockets of Balb/c mice. Implants were studied after removal and it was found that ectopic bone formation was similar in both scaffolds. However, ectopic bone formation in scaffolds loaded with SCF-1 showed a statistically significant increase in capillary formation. The results indicate that cell-homing strategies such as this must be further explored, because it reduces the complications of cell seeding and increases angiogenesis, a requirement for the formation of healthy bone.

Hyaluronic Acid

HAc has also demonstrated potential as a bone scaffold material. It is naturally occurring, hydrophilic, and nonimmunogenic, and has been found in the cytoplasm of osteoprogenitor cells [48]. This natural polymer has been used in combination with other materials, factors, and drugs to enhance its osteogenic potential. HAc, a nonsulfated glycosaminoglycan present in all connective tissue as a major constituent of the ECM and particularly prevalent during wound healing, has been proposed for the preparation of biodegradable ECM-like constructs for tissue engineering applications [49]. The ECM is a hydrophilic matrix with a gel-like uniformity consisting primarily of collagen fibers, proteoglycan filaments, and entrapped interstitial fluid. Similarly, hydrogels are also usually composed of covalently cross-linked hydrophilic polymers that enable them to swell while retaining their 3D structure without dissolving. This results in scaffolds structurally similar to the ECM, with a high water content and excellent biocompatibility characteristics. Cross-linking of HA forms experimentally controllable hydrogels that

provide a microstructure similar to native ECM. Subsequent encapsulation of cells into permeable HA hydrogels thus provides both structural support and protection, as well as the ability for cells to interact in 3D, which has been shown to increase the viability of transplanted cells significantly [50]. Furthermore, cell encapsulation with biocompatible materials such as HA has been shown to reduce the immunogenicity of the tissue engineered construct, because biocompatible materials reduce the adsorption of proteins [51], which would normally stimulate the recruitment of immune cells such as macrophages.

In one study, a photocured HAc hydrogel containing an osteogenesis-inducing drug, simvastatin (SIM), was designed [52]. SIM was found to induce osteodifferentiation of human adipose-derived stromal cells. Hydrogel viscoelastic properties were fine-tuned through 2-aminoethyl methacrylate (AEMA) substitution (HAc-AEMA). Three different HAc-AEMA scaffolds were studied: HAc with 20% (wt/wt) AEMA (HAc-AEMA-20), 30% AEMA (HAc-AEMA-30), and 40% AEMA (HAc-AEMA-40). Rheological measurements showed that elastic storage of the hydrogel increased with increasing AEMA concentrations (from 40 to 80 Pa). Pore size increased with increasing AEMA concentration, but sizes were not reported. MC3T3 fibroblasts were seeded into the hydrogels to test for cytocompatibility; it was found that the material was cytocompatible and that there was no difference in viability between groups. After SIM was loaded into the hydrogel, release kinetic experiments showed that it could sustain release for up to 14 days. HAc-AEMA-40 hydrogel was chosen for the remaining experiments. Loading of 1 mg SIM into HAc-AEMA-40 significantly increased fibroblast proliferation and mineralization at all times tested. The presence of SIM also upregulated the expression of OCN and OPN at all times tested. The effectiveness of this system was then tested in vivo with and without SIM. Hydrogels were implanted in parietal bone defects in rabbits. Cone beam computed tomography was used to assess healing of the bone defect for up to 9 weeks. Healing was only slightly superior at 9 weeks in the SIM-containing hydrogel. These results showed that substitution with AEMA can improve viscoelastic properties and the addition of SIM into the hydrogel improved osteogenesis in vitro, although the results were not as notable in vivo.

Alginate

Alginate–HA composite scaffolds were prepared by internal gelation followed by freeze-drying to obtain a porous structure. The nanoparticles were prepared in presence of a lactose-modified CHI; this colloidal solution was adsorbed on the scaffolds by exploiting electrostatic interactions and was used as temporary resorbable bone implants [53]. A CHI–polypyrrole–alginate composite scaffold can act as a substrate for tissue regeneration and can be employed for bone tissue engineering using osteogenic cells by employing electrical stimulation with a bioreactor system, and thus evaluating the role of conducting substrate in bone regeneration [54].

CHI–alginate hybrid scaffolds displayed improved mechanical strength and structural stability and were shown to stimulate new bone formation and rapid vascularization [55]. CHI–alginate gel–MSC–BMP-2 composites should have become clinically useful injectable materials to generate new bone [56]. Porous HA–CHI–alginate composite scaffolds were prepared through in situ coprecipitation and freeze-drying for bone tissue engineering [57]. Two different types of polymer scaffolds, such as CHI–alginate and CHI–alginate with fucoidan, were developed by freeze-drying; each was characterized as a bone graft substitute by Venkatesan et al. [58]. Alginate microparticle and microfiber aggregated scaffolds were produced with alginate through the aggregation method. Such a porous structure will allow vascularization, oxygenation and cell migration, adhesion, and proliferation, which are biological events that are fundamental for bone tissue regeneration [59].

In another study, macroporous alginate scaffold was fabricated and mineral-coated using a biomimetic approach [60]. The nucleation of HA was achieved by incubating the scaffold in modified simulated body fluids for up to 4 weeks. Mineralization of the scaffold was determined by a change in mass, which increased steadily from weeks 1 to 4. Energy-dispersive X-ray spectroscopy revealed a Ca/P ratio of 1.61 when the Ca/P ratio of pure HA (HAP) was 1.67. Viability experiments demonstrated that the HAP coatings supported the attachment and proliferation of hMSCs. The cell number was significantly higher in the coated scaffolds at all times tested. This work showed that a simple scaffold modification, such as immersion in simulated biological fluids, can change the topography and environment of a scaffold to improve the osteogenic outcome of the scaffold. The in vivo application of a facile polyelectrolyte complexation (PEC) process was employed to condense heparin onto the surfaces of poly-L-ornithine (PLO), poly-L-arginine (PLA), and diethylaminoethyl–dextran-coated alginate microbead templates that entrap bioactive recombinant human BMP-2 (rhBMP-2). In vivo implantation of PEC shells loaded with rhBMP-2 resulted in new bone formation that could stimulate a mechanically stable posterolateral spinal fusion in rats (PLO- and PLA-based PEC shells). This ability to retain or regulate rhBMP-2 delivery and stimulate new bone formation from heparin-incorporated PEC shells could provide a powerful tool for entrapping and controlling the delivery of several growth factors in a variety of bone tissue engineering [61].

Chitosan

CHI, the deacetylated form of chitin, is the structural component found in the exoskeleton of crustaceans such as shrimp, crabs, and lobsters. It is a natural polymer, with a linear structure consisting of β(1−4)glycosidic bond−linked D-glucosamine residues with a variable number of randomly located *N*-acetyl-D-glucosamine groups [62]. CHI is bioactive, biodegradable, antibacterial, and biocompatible and possesses a hydrophilic surface, which is absent in many synthetic polymers. Here we describe several studies in which the osteogenic properties of CHI 3D scaffolds were modified by adding other polymers, cells, and bone-inducing factors. CHI and poly(butylene succinate) scaffolds were constructed and seeded with human MSCs to test their ability to induce osteogenesis [63]. The material was found to have 59% porosity, a 144.9-mm pore size, and 60.9% interconnectivity. MSC viability increased for 21 days in the scaffold and ALP activity also increased for 21 days after MSCs were exposed to osteogenic induction. The ability of the MSC-seeded scaffolds to repair a cranial critical-sized bone defect in mice was also examined. The cell−scaffold constructs were cultured in osteogenic-induced medium for 2 weeks before implantation. Cell-free scaffolds were used as a control. The crania were observed 8 weeks after implantation. Bone formation was analyzed using μCT. The results showed an elevated rate of bone formation in the cell-seeded scaffold, evidencing the importance of the existence of stem cells near the area where bone formation is needed. In another study, human bone marrow−derived stem cells (hBMSC) were encapsulated in hydrogels at CHI-collagen ratios of 100/0, 65/35, 25/75, and 0/100 wt%. β-Glycerophosphate was added to hydrogels, because it has been shown to be an osteogenic supplement when added to cultures of hBMSC, and it has also been used as a catalyst to sol-gel transitions in CHI hydrogels. The effect of adding collagen to CHI on matrix mechanical properties was assessed. Stress−strain profiles (0%−8% strain) showed that all collagen materials were approximately three times stiffer than pure CHI, which has a modulus of 6.3 kPa. When evaluating cell proliferation, the DNA content dropped by about half over 12 days in pure CHI materials whereas it increased twofold in materials containing collagen. For these reasons, only collagen-containing materials were examined for their effect on osteogenic gene expression. hBMSCs were encapsulated in CHI-collagen and collagen hydrogels and exposed to osteogenic medium. Hydrogels with a CHI-collagen ratio of 65/35 had the highest levels of osterix expression, bone sialoprotein expression, and ALP activity. These osteogenic markers started to decrease at lower CHI concentrations. These results suggest that the presence of collagen was highly beneficial for the osteogenic capabilities of the 3D scaffold, although it remains to be determined whether this was caused by the change in mechanical properties or the intrinsic biological properties of collagen.

Another research group prepared porous 3D scaffolds based on CHI, CHI/SF, and CHI/SF/HA. SF/HA scaffolds were previously reported to be unsuitable for bone tissue engineering owing to insufficient formability and inflexibility [64]. The CHI scaffolds had a porosity of 94.2% ± 0.9%, which was statistically higher than the one presented by CHI/SF/HA scaffolds, which had a porosity of 89.7% ± 2.6%. The CHI/SF scaffold had a porosity of 91.6% ± 1.2%, which was not significantly different from other materials. SaOs-2 cells were used to measure viability and differentiation. At day 21, there was a statistically significant increase in cell proliferation and ALP activity in the CHI/SF/HA scaffolds compared with CHI and CHI/SF. It is unclear whether it was the presence of HA or the changes in porosity that promoted osteogenesis.

CHI has also been used as an injectable biomaterial. Bi et al. [65] produced an injectable composite of tricalcium phosphate (TCP), CHI, and platelet-rich plasma (PRP). A TCP-CHI composite (TC) was used as a control. PRP contains a number of growth factors (i.e., PGDF, transforming growth factor-β, insulin-like growth factor, basic fibroblast growth factor [bFGF], and VEGF), which have often been shown to have an important role in bone tissue engineering applications. The composites were fabricated into cylinders for mechanical testing. It was found that the compressive strength (MPa) of both composites increased over time (∼9−16 over 7 days) but there was no difference between them. The MSCs were seeded onto TCP and TC scaffolds and cell proliferation was measured over 7 days; the number of cells on TCP was found to be significantly higher than on TC at every time point tested. MSCs grown in plates in osteogenic media (OM) were used as a positive control for osteodifferentiation analysis of the materials being tested. ALP activity was tested at 7 and 14 days. At 7 days, ALP activity was higher in PTC than TC, and PTC and OM had comparable levels. At day 14, ALP activity was significantly higher in PTC than TC and even OM. RT-PCR analysis was used to study the expression of osteogenesis-related genes. Runt-related transcription factor 2 (Runx-2), type-1 collagen and osteonectin expression in TCP were comparable to those in OM.

Peptide Hydrogels

Hydrogels are 3D networks composed of hydrophilic polymers cross-linked through covalent bonds or held together via physical intramolecular and intermolecular attractions. Hydrogels can absorb huge amounts of water

or biological fluids, up to several thousand percent, and readily swell without dissolving. The high hydrophilicity of hydrogels results from the presence of hydrophilic moieties such as carboxyl, amide, amino, and hydroxyl groups distributed along the backbone of polymeric chains. In the swollen state, hydrogels are soft and rubbery, resembling living tissues to a great extent. In addition, many hydrogels such as CHI and alginate-based hydrogels have desirable biocompatibility [66], can easily be modified to contain bioactive motifs and composed of self-complementary amphiphilic peptides, and when gelled they provide a 3D structure that has many similarities to the ECM. In one study, self-assembling peptide nanostructured gels were constructed using peptide amphiphile (PA) materials with or without phosphoserine residues near their surfaces and with or without Arg-Gly-Asp-Ser (RGDS) peptide [67]. The phosphoresidues contribute to mineralization and the RGDS peptide contributes to cell adhesion. The authors proposed that the architecture of these PA materials is highly biomimetic of the fibrous elements commonly found in ECM, such as collagen fibrils. The results of this study suggested that a 3D matrix that supports mineralization and cell adhesion is favorable for bone formation, but the outcome was not superior to implantation with demineralized bone tissue. In another study, the commercially available peptidic hydrogel PuraMatrix was evaluated as a candidate to assess its potential for the osteogenic differentiation of dedifferentiated fat cells (DFATs) [68]. PuraMatrix is composed of 8–16 L-amino acid residues and forms a 3D scaffold that is biocompatible and biodegradable. The cell suspension was mixed at a 1:1 ratio with the hydrogel. These results indicate that commercially available PuraMatrix can support the osteogenic differentiation route and that DFATs are a viable alternative to MSCs that should further be explored with regard to bone regeneration.

Synthetic Polymers

Copolymers

A range of polyethylene oxide (PEO)–polybutylene terephthalate (PBT) copolymers (70%–30% PEO) was investigated for non–load bearing bone replacement. In general, copolymers are attractive for tissue engineering applications because their physicochemical properties are highly controllable. Gel formation dynamics, cross-linking density, and material mechanical and degradation properties can be controlled by regulating molecular weights, block structures, degradable linkages, and cross-linking modes [69].

β-Keto nitrile tautomeric copolymers have demonstrated fine-tunable hydrophilicity and hydrophobicity properties according to their surrounding environment and mechanical properties similar to those of human bone tissue. These characteristic properties make them promising candidates as biomaterials for bone tissue engineering. Based on this knowledge, we designed two scaffolds based on β-keto nitrile tautomeric copolymers that differ in chemical composition and surface morphology. Two of them were nanostructured, using an anodized aluminum oxide template and the other two were obtained by solvent casting. They were used to evaluate the effect of the composition and their structural modifications on the biocompatibility, cytotoxicity, and degradation properties. The results showed that the nanostructured scaffolds exhibited a higher degradation rate by macrophages than the casted scaffolds (6% and 2.5% of degradation for the nanostructured and casted scaffolds, respectively), a degradation rate compatible with bone regeneration times. We also demonstrated that the β-keto nitrile tautomeric–based scaffolds supported osteoblastic cell proliferation and differentiation without cytotoxic effects, which suggested that these biomaterials could be useful in bone tissue engineering [70]. In one study, the authors had developed a novel BMP-2–related peptide (P24) that was shown to enhance the osteoblastic differentiation of BMSCs. The purpose of that study was to incorporate P24 into a modified PLGA-(PEG–ASP)n copolymer to promote bone formation [71]. PLGA and PLGA-(PEG–ASP)n membranes were fabricated by solvent coating and evaporating. In in vitro release studies, about 70% of peptide was released after 14 days. Adhesion and proliferation studies were performed with BMSCs and the PLGA-(PEG–ASP)n scaffolds, with and without p24, promoted cell adhesion and proliferation compared with the PLGA control. There was no difference between PLGA-(PEG–ASP)n and PLGA-(PEG–ASP)n-p24 scaffolds in either cell adhesion or proliferation. However, in cell differentiation studies, the PLGA-(PEG–ASP)n-p24 scaffold was shown to enhance differentiation at each time point tested for up to 20 days, as determined by ALP activity.

A novel three-component biomimetic hydrogel composed of triblock PEG–PCL–PEG copolymer (PECE), collagen, and nano-HA (n-HA) was successfully prepared [72]. The in vivo biocompatibility of the PECE–collagen–n-HA hydrogel composite was tested by implanting the composite into the dorsal muscle pouches of Wistar rats for 3, 7, and 14 days. Some inflammation occurred during the degradation process, but by day 14, the inflammatory response disappeared completely. The activity of bone regeneration was evaluated by reconstructing two rectangular defects in rabbit craniums. The untreated left defects were used as control and the right defects were packed

with PECE−collagen−n-HA hydrogel composite. At 20 weeks, as determined by histological staining, the right defect was filled completely with new bone and the amount of high-density tissue in the control defect was visibly less than the treatment defect. In another study, biodegradable and electroactive poly(ester amide)s containing conjugated segments of phenyl amino end-capped tetraaniline (PEA-g-TA) were prepared [73]. Composites were prepared under different feed weight percentages of tetraaniline (TA) (3.5%, 8.4%, and 15.5%). The copolymer solutions were cast onto a superflat polytetrafluoroethylene plate and placed for 5 h under room temperature to form thin films. Conductivity increased with an increasing percentage of TA (7.11×10^{-7}, 8.01×10^{-6}, and 2.45×10^{-6} S/cm for 3.5%, 8.4%, and 15.5%, respectively) but all scaffolds had conductivity values adequate to transfer bioelectrical signals in vivo. The PEA-g-TA number 2 copolymer (8.4% feeding ratio) was highly distensible with a breaking elongation rate of 105% ± 10%, and the tensile modulus of copolymer was 20 ± 2.5 MPa. These values were not reported for the other scaffolds. Mouse MC3T3-E1 cells showed more than 90% viability in all copolymers tested. Cells were stimulated by pulsed electrical signal for 2 hours every day for 14 days. Osteogenic differentiation of MC3T3-E1 cells was assessed by the intracellular free calcium concentration and ALP enzyme activity. After 14 days, there were no significant differences between the materials tested; however, cells that were stimulated by the electric signal had higher intracellular calcium concentration and ALP enzyme activity. This work illustrates that combining appropriate materials with physical stimulation such as electrical impulses can produce superior responses.

Polyesters

Polyphosphazene−polyester blends are attractive materials for bone tissue engineering because of their controllable degradation pattern with nontoxic and neutral pH degradation products. Aliphatic polyesters such as polyglycolic acid, polylactic acid, and polycaprolactone are the most commonly used polyesters for tissue engineering. Their degradation products are present in the human body and can be removed by natural metabolic pathways. 3D scaffolds from these materials can be fabricated through various techniques and fine-tuning the molar ratios of these polymers can influence mechanical properties and degradation rates [74,75]. In one study, PCL scaffolds incorporating HA particles were fabricated by combined solvent casting and particulate leaching [76]. The presence of HA slightly increased the density of the scaffold but had no significant effect on the porosity values of the scaffolds. With increasing HA concentrations, there was a significant correlation with an increase in the compressive modulus. Viability assays showed that the number of primary human bone cells that had been cultured on the PCL−HA scaffolds was greater than that of cells that had been cultured on the neat PCL scaffolds at both 24 and 48 h after cell culturing. Expression levels of type I collagen and osteocalcin were evaluated by RT-PCR and were significantly greater in cells cultured on the PCL−HA scaffolds than those in cells cultured on the neat PCL scaffolds on day 10. The formation of mineralized nodules of cells cultured on the PCL−HA scaffolds was significantly greater than that of cells cultured on the neat PCL scaffolds. For in vivo experiments, a circular calvarial defect in mouse was used as a model. Six weeks after implantation, histomorphometric analysis indicated a statistically significant increase in the amount of new bone formation in the PCL−HA scaffolding implants compared with neat PCL counterparts.

Ceramic Scaffolds

Bioactive ceramics have received great attention owing to their success in stimulating cell proliferation, differentiation, and bone tissue regeneration. They can react and form chemical bonds with cells and tissues in human body. These ceramics are recognized as bioceramics and are classified into two groups: bioinert or bioactive. Bioactive ceramics are categorized as resorbable or nonresorbable [77]. Ceramics are used because of their chemical properties and crystallinity, which is similar to bone mineral components. These materials exhibit excellent biocompatibility and bioactivity. The inorganic fraction of bone is composed of HA and CaPs, which allow the formation of bone tissue on its surface. This type of material is excellent as an implant, but it has particular problems with its mechanical properties in terms of fracture and fatigue. Common ceramic materials used for bone repair or regeneration are Bioglass, CaPs, and ceramic scaffold derived from corals. Here, we report some studies in which these materials were studied both in vitro and in vivo to assess their osteogenic potential.

Bioactive ceramics are known to enhance osteoblast differentiation as well as osteoblast growth. However, their clinical applications have been limited because of their brittleness, difficulty in shaping, and extremely slow degradation rate in the case of HAP. Also, they have poor fidelity and reliability, and new bone formed in a porous ceramic scaffold cannot sustain the mechanical loading needed for weight-bearing bone [78]. Wang and Shaw [79] reported that dense HAP ceramics with a fracture toughness of 0.61−1.06 MPa·m1/2 were fabricated via conventional sintering. Fielding et al. [80] fabricated TCP scaffolds with a compressive strength of 1.75−5.48 MPa using commercial 3D

printing technology. However, the mechanical properties of fabricated scaffolds were far below those of weight-bearing bone (fracture toughness = 2−12 MPa·m1/2; compressive strength = 130−180 MPa) in the human body [81]. Therefore, obtaining an effective method to overcome these limitations has become the focus of current and future research in bone tissue engineering.

Calcium Phosphate

Calcium phosphate ceramics (CPCs) are a class of fine-tunable bioactive materials that have been widely used for bone tissue repair and augmentation. They possess surface properties that support osteoblast adhesion and proliferation (i.e., osteoconduction) and stimulate new bone formation (i.e., osteoinduction) [82]. TCP was first used in 1920 by Albee [83]. The study reported cases of fracture with bone loss in which more rapid bone growth and union were observed when TCP was injected into the gap between the bone ends than did the controls without its use. Also, it was demonstrated that osteogenesis was stimulated by this material in conjunction with the fracture microenvironment. In 1951, Ray and Ward [84] reported the use of granular synthetic HA to repair defects in long bone and iliac wings of dogs and bur holes in the crania of cats; the study demonstrated that this material could be replaced by new bone, but it was not as effective as autologous bone grafts in repairing the defects. Calcium phosphate−based scaffolds exhibit osteoconductivity, bioactivity, and resorbability in vivo owing to their complex chemical composition (Ca/P ratio) and physical properties such as their crystallographic structure and porosity [85]. In studies combining CPC paste and hydrogel microbeads to encapsulate human umbilical cord MSCs (hUCMSCs) with CHI fibers [86], hUCMSC viability and differentiation capacity were observed.

Nanostructured CaP biomaterials and scaffolds mimic natural bone, and have high surface-to-volume ratios, improved wettability and mechanical properties, and increased protein adsorption and other desirable properties, compared to conventional counterparts. Nano-CaP biomaterials have emerged as a promising class of biomimetic and bioactive scaffolds capable of directing cell behavior and cell fate and enhancing tissue formation in vivo. In general, nano-CaP scaffolds can support stem cell attachment and proliferation and induce osteogenic differentiation, in some cases without osteogenic supplements. The influence of nano-CaP on cell alignment is less prominent than that of polymers and metals owing to the no-uniform distribution of the nano-CaP crystals. Nano-CaP biomaterials can achieve significantly better bone regeneration in vivo than conventional CaP biomaterials. The combination of various types of stem cells with nano-CaP scaffolds can further accelerate bone regeneration, the effect of which can be even further promoted by growth factor incorporation. Cell microencapsulation combined with nano-CaP scaffolds is a promising tool for bone tissue engineering applications to distribute cells throughout the interior of the scaffold [87]. More studies are needed to compare various types of nano-CaP compositions and nanostructures side by side in vivo and to compare the efficacy of various types of stem cells in bone regeneration.

Bioglass

Since the discovery of 45S5 bioactive glasses by Hench, they have been frequently considered scaffold materials for bone repair [88]. The need to find a material that forms a living bond with tissues led Hench to develop Bioglass repair tissues during the Vietnam War [89]. Bioglass offers advantages such as a controlled rate of degradation, excellent osteoconductivity, bioactivity, and the capacity to deliver cells, but they have limitations in certain mechanical properties such as low strength, toughness, and reliability [90]. Advantages of the glasses are ease in controlling the chemical composition and thus, the rate of degradation, which make them attractive as scaffold materials. The structure and chemistry of glasses can be tailored over a wide range by changing the composition or the thermal or environmental processing history. Therefore, it is possible to design glass scaffolds with variable degradation rates to match those of bone ingrowth and remodeling. A limiting factor in using bioactive glass scaffolds to repair defects in load-bearing bones has been their low strength [91]. Work has shown that by optimizing composition, processing, and sintering conditions, bioactive glass scaffolds can be created with predesigned pore architectures and with strength comparable to that of human trabecular and cortical bones [92]. Another limiting factor of bioactive glass scaffolds has been the brittleness. This limitation has received little interest in the scientific community, judging from the paucity of publications that report on properties such as fracture toughness, reliability (i.e., Weibull modulus), or work on the fracture of glass scaffolds.

Wu et al. [93] prepared 45S5 Bioglass by foaming with rice husks and sintering at a high temperature; they obtained favorable results in compressive testing and degradability in simulated body fluid. That study reported compressive strength values in the range of trabecular bone. Porous bioactive glass-ceramic (45S5) was tested with human umbilical vein endothelial cells (HUVECs) and human osteoblast-like cells (HOBs). The results of the study demonstrated that the proliferation of HOB and HUVEC cocultures seeded on scaffolds was higher than that of commercial HA scaffolds. Wu et al. [93] studied HOB attachment, proliferation, and differentiation

on a bioactive diopside scaffold ($CaMgSi_2O_6$). Cell proliferation increased with incubation time. In addition, porous $CaSiO_3$ and kermanite ($Ca_2MgSi_2O_7$) ceramic scaffolds were compared with β-TCP ceramic scaffolds, demonstrating in vivo bone formation and potential applications in bone tissue regeneration [94]. Other studies related to the formation of porous Bioglass were developed by Moawad and Jain [95]. They fabricated nanomacroporous soda lime phosphosilicate glass scaffolds using sucrose as a macropore former, and established process parameters such as the weight ratio of glass/sucrose, the particle size of glass/sucrose powders, and the time and temperature of sucrose dissolution.

Coral

Coral exoskeleton ($CaCO_3$), which has an interconnected pore structure that resembles that of natural human bone, has been used as a scaffold material to fill bone defects in both animal models and humans since the early 1970s. This natural material is biocompatible, osteoconductive, and biodegradable. Most important, the possibility of seeding coral scaffolds with stem cells or loading them with growth factors has provided a novel alternative for bone tissue engineering. Corals are attractive materials for scaffolds because they have microstructures with highly controlled pore sizes and an interconnected porous architecture similar to trabecular bone [96,97]. After 1991, Ripamonti reported the first study of the morphogenesis of bone in porous bioceramics (coral-derived HA) in the rectus abdomen in the muscle of adult nonhuman primates Papioursinus demonstrated the use of coral line replicas of HA with an average pore size of 600 μm as bone grafts for the controlled formation of bone in humans [98]. Coral scaffolds using adipose-derived MSCs (AMSCs) have been used to repair cranial bone defects in a canine model [99]. They showed adipogenic differentiation and good biocompatibility to support the proliferation of AMSCs in vitro. Subsequent implantation in vivo showed new bone formation. Certain types of proteins such as ALP, OPN, and OCN are employed to identify the activity of osteoblast-specific proteins. Suzina et al. [100] reported an increase in the expression of specific genetic markers from osteoblasts such as Runx-2, OPN, ALP, and OCN in the *Porites* Goniopora coral, which was implanted in orthotropic calvarial defects of the adult nonhuman primate *Papio ursinus*. In vivo results showed an increase in ALP activity from 2 to 12 months and induction of bone in the concavities; however, limited conversion of HA−calcium carbonate was observed.

A preliminary study in nude mice reported the vascularization of tubular coral scaffold with cell sheets [101]. The results showed that cells promoted new bone formation through an endocrine process. In addition, Zheng et al. [102] evaluated the feasibility of mandibular condyle constructs engineered from hBMSCs. In vitro studies reported that hBMSCs induced differentiation into osteoblasts and chondroblasts, and seeded scaffolds implanted into nude mice showed neovascularization in the temporomandibular joint detected by bFGF expression.

Metallic Scaffolds

Several biocompatible metallic materials are frequently used as implanting materials in dental and orthopedic surgery to replace damaged bone or provide support for healing bones or bone defects. Standard surgical implant materials include stainless steel 316 L (ASTM F138), Co-based alloys (mainly ASTM F75, and ASTM F799), and titanium alloys; Ti−6Al−4V (ASTM F67 and F136) is the most commonly employed. However, the main disadvantage of metallic biomaterials is their lack of biological recognition on the material surface. To overcome this restraint, surface coating or surface modification offers a way to preserve the mechanical properties of established biocompatible metals improving the surface biocompatibility. In 1909, the first patent of a metallic framework for an artificial tooth root for fixation by bone in growth was accredited to Greenfield [103,104]. He recognized the limitations of natural tooth implantation and started experimenting with implanting artificial hollow cylinders made of iridoplatinum wire soldered with 24 kt gold. In 1971, Galante et al. [105] were pioneers in developing open-pore fiber metals for clinical use as porous coatings in hip and knee arthroplasty. Because metals are materials with high mechanical strength and fracture toughness, they are frequently used as metallic biomaterials in the dental and orthopedic fields to replace and offer support for damaged and healing bone [106,107]. The commonly metals used as standard surgical implants were stainless steel 316 L (ASTM F138), Co-based alloys (mainly ASTM F75, and ASTM F799), and titanium alloys. where Ti−6Al−4V (ASTM F67 and F136). However, these metallic biomaterials have disadvantages such as the possible release of toxic metallic ions and/or particles through corrosion or wear processes that cause inflammation and allergic reactions, which affect biocompatibility and tissue loss. Also, they produce poor stimulation of new bone growth owing to the elastic moduli, which does not correspond with natural bone tissue. Despite this, it has been reported that Ti-based metals can be used as bone substitute because of its elasticity, mechanical properties, shape memory effect, porous structure, and biocompatibility [108]. Also, 3D microporous NiTi and Ti scaffolds have been produced by powder metallurgy that obtains a hydrophilic surface to facilitate the deposition of HA and stimulate cell attachment and proliferation.

Hybrid Materials

The material needs to be bone bioactive: that is, it should encourage bone ingrowth and degrade at a rate that allows the newly formed tissue to replace the scaffold gradually, both as a mechanical structure and in terms of the space occupied. Finally, and this is where most current materials fail, the material needs mechanical properties that allow the device to be implanted without failing. This last requirement allows a patient to use the implanted area without mechanical protection, such as a cast, but still enables sufficient loading of the newly formed tissue to stimulate the osteoblasts mechanically. As yet no one has reported a material that fulfils all of these requirements. One group of materials that attempts to fulfill many of these requirements is composites of degradable polymers reinforced with ceramics, glass-ceramics, or bioglasses. If the polymer is biodegradable and the ceramic, glass-ceramic, or bioglass phase is degradable or metabolized by the body, the degradation requirement is fulfilled. The use of ceramics or glass-ceramics can both stiffen and strengthen a low-modulus, low-strength polymer and increase the bioactivity of the composite [109]. Commonly, composite scaffolds are fabricated using a different type of matrix with a dispersed phase such as polymer−ceramics, ceramic−metals, and polymer−metals [110].

Polymer−Polymer Blends

A polymer−polymer blend defines a mixture of two different polymers addressing each other's drawbacks and giving synergetic advantage to the scaffold design. A miscible composite design with desired features can be fabricated by employing polymers with particular intermolecular or van der Waals interactions. One important example is PLGA and polyphosphazene bends [111]. PLGA is a well-known biomaterial in tissue engineering. Owing to PLGA acidic by-products upon degradation, it has been a critical issue for its further use, because its long-term tissue exposure to acidic products may lead to tissue necrosis and implant failure. On the other hand, polyphosphazene releases neutral or basic products upon degradation. Thus, PLGA has been blended with a wide arena of polyphosphazenes to achieve near-neutral degradation products as an efficient construct for tissue engineering [112−114].

Polymer−Ceramics Blends

Many researchers have studied polymer−ceramic-based scaffolds. Combinations of CaP−polyesters for scaffold production were investigated by Kaplan and coworkers [115], who developed a scaffold from SF−poly aspartic acid coated with CaP. In vitro studies showed cell viability, proliferation, and osteogenic differentiation; however, nonuniform distribution of cells resulting from mineral deposition was observed. Li et al. [115] used two biocompatible and biodegradable polymers, PLGA and PCL, with a layer of CaP and a gelatin coating. MC3T3 cells adhered to regions with higher CaP content along the scaffold, which indicated that the mineralization gradient affects the adhesion and proliferation of cells and the physical properties of the scaffold. Relative to the mechanical properties, the local strain varied along the long axis of the scaffold and the Young's modulus increased with increasing levels of CaP.

TCP has been used to generate biocompatible and biomechanical scaffolds [116]. In vivo studies using this type of scaffold reported significant biocompatibility, sufficient mechanical strength, osteogenic differentiation, and bone growth. It was also demonstrated that the incorporation of collagen into this type of system improves hydrophobicity and differentiation [117]. Wang et al. [118] reported a novel scaffold composed of the PLGA−β-TCP skeleton wrapped with type I collagen. The aim of that study was to analyze the physical properties and biocompatibility of the core−sheath structure composite scaffold in vitro compared with the PLGA−β-TCP skeleton. No differences were observed in the porosity ratio, compressive strength, or Young's modulus of the scaffolds. MTT assay indicated that BMSCs showed better adhesion and proliferation activity on the surface of the core−sheath structure composite scaffold than on the PLGA−β-TCP skeleton. In a previous study [119], this scaffold was fabricated via low-temperature deposition manufacturing but its hydrophobicity did not promote adequate cell adhesion, proliferation, or osteoblastic differentiation. Thein-Han and Xu [120] were the first to develop a novel scaffold from collagen−CPC using hUCMSCs for in vitro analysis. Good mechanical properties, hUCMSC attachment, viability, and osteogenic differentiation were observed. Previously, studies were conducted using collagen−CaP scaffolds, but they were not moldable or injectable [121]. HA was also employed to fabricate ceramic−polymer scaffolds [122−124]. Kim et al. [125] developed this type of scaffold and demonstrated that the combination of PGA−PLGA−HA improves their osteogenic capacity. Hollinger et al. [125] used a combination of poly(ε-caprolactone fumarate) −PVA−HA with rhBMP-2 and the preosteoblast cell line MC3T3-E1 obtained good cell viability and proliferation, bioactivity, and bone regeneration and an increase in compressive modulus with an increase in HA; however, μCT showed a nonuniform distribution of the porogen indicating that the degradation process, porosity, and pore

interconnectivity needed to be improved. Geng et al. [126] fabricated a scaffold from a magnesium phosphate (MP)−poly(ε-caprolactone) composite in which the mechanical analysis showed low values of the compressive modulus compared with trabecular bone; thus, this scaffold was found not to be appropriate for high−load bearing applications. It was also reported that the degradation rate of the composite scaffolds could be modulated by varying the amount of MP particles introduced into the polymer matrix.

Metal−Polymer Blends

The development of successful scaffolds for bone tissue engineering requires a concurrent engineering approach that combines different research fields. Researchers have tailored metallic scaffolds that are useful for a wide variety of medical and dental applications. Surface modification of already proved biocompatible metals is an essential requisite for their use in tissue engineering because the metal surface must be controlled to induce the adhesion and proliferation of cells and the adsorption of essential biomolecules. Chemical and physical properties have a crucial role in the osteointegration of implant surface; they allow protein adsorption between implanted biomaterials and the biological environment. Thus, numerous strategies have been developed to create a bond between the implant and the living host tissue. The use of metallic implants with a polymer coating has been reported by many for these purposes. Ti, TiO_2, and Ti-alloy combined with polyester coatings have been intensively studied to fabricate scaffolds. Lagoa et al. [127] developed a partially biodegradable implant from titanium, polylactide, HA, and calcium carbonate. The implant had mechanical stability, biocompatibility, and partial biodegradability. Helary and coworkers used poly(sodium styrene sulfonate) (polyNaSS) on oxidized or grafted Ti samples [128] and polyNaSS−(methylacrylic acid) MA grafted onto Ti_6Al_4V alloy surfaces [129] They reported that cell adhesion and differentiation on Ti (grafted) were higher than on oxidized titanium and titanium because of the presence of active sites that interact with extracellular proteins. In addition, polyNaSS−MA grafted onto Ti6Al4V alloy in femoral rabbit model showed lamellar trabecular bone with wide haversian canals lined by osteoblasts; however, the high levels of Na, P, Ca, Zn, etc., indicated that the piranha treatment used to oxidize the alloy surface was intense. Oughlis et al. [130] developed a scaffold from polyNaSS polymer and titanium. The results showed cell viability, proliferation, and osteoblastic differentiation of hMSCs on this scaffold.

Metal−Ceramic Blends

Many researchers have reported the development of metal−ceramic scaffolds that have been shown to possess favorable characteristics regarding their mechanical properties and bioactivity (cell attachment, proliferation, and differentiation). Yang et al. [131] developed a biodegradable and bioactive scaffold composed of magnesium with a coating of β-TCP. The in vitro results showed good cell adhesion and bioactivity. In similar studies, when tantalum and titanium were used as metals, osteoconductivity and osteoinductivity were improved in vivo. Wu et al. [132,133] reported the fabrication of a hypoxia-mimicking mesoporous bioactive glass (MBG) scaffold by incorporating Co^{2+} ions into the MBG scaffold. Results showed that MBG scaffolds had no significant cytotoxicity and that the incorporation of ionic Co^{2+} ions enhanced VEGF secretion, HIF-1a, expression, and bone-related gene expression in BMSCs. Also, the CoMBG scaffolds supported BMSC attachment and proliferation. It was reported that ZrO_2 itself does not have good cellular and tissue affinity [134] although in vitro and in vivo studies demonstrated that ZrO_2 is not toxic [135]. Lee et al. [136] fabricated a scaffold composed of (biphasic CaP) ZrO_2 and PCL layers. The scaffold had a compressive strength of 12.7 MPa and a porosity of 78 vol% and showed excellent MG63 cell attachment, and OCN and collagen expression. It was also shown that PCL incorporated into biphasic CaP gave scaffolds high biodegradability, cell attachment, and proliferation.

Li et al. [137] reported a novel ion doping method applied in calcium polyphosphate (CPP)-based bioceramic scaffolds to be substituted by potassium and strontium ions (K/Sr). The K/Sr CPP scaffolds had a higher compressive strength, cell biocompatibility, biodegradability, osteoinductivity, and osteoconductivity and better degradation properties than the pure CPP scaffolds; however, the mechanical strength of K/Sr-CPP was not good after degradation. Studies suggested that directly mixing Cu^{2+} ions with bioactive materials improves angiogenesis [138]. Xiao et al. (2013) prepared copper (Cu)-containing mesoporous bioactive glass (Cu-MBG) scaffolds with interconnected large pores. Attachment, proliferation, and ALP activity of hBMSCs on Cu−MBG scaffolds were observed, and ionic products of Cu−MBG extracts enhanced the osteogenic differentiation of hBMSCs. Studies using porous ceramic−coated TiO_2 as scaffolds were reported by Dimitrievska et al. [139]. They evaluated the cell adhesion, growth, and osteoblastic differentiation of hMSCs in the TiO_2−HA nanocomposite; however, applications in bone implants were limited by their bioinertness. Also, Haugen et al. [140] reported in vitro results that showed the cell viability of the mouse osteoblastic cell line MC3T3-E1. No cytotoxic effects from the TiO_2−HA scaffolds were found. It was also reported that healing occurred when this scaffold was implanted into rabbit defects.

CONCLUSIONS AND FUTURE PROSPECTS

We have summarized the current state of the art of materials technology and the development requisites for successful bone graft substitute. Clearly, the smart selection of materials is the key to successful tissue engineering. Nevertheless, various other factors also influence the overall outcome. Scaffold composed of natural or synthetic biomaterials or their combination should closely mimic the ECM in native tissues, provide the required support, promote neovascularization, and allow access to nutrients to support the entire process of tissue regeneration. All of these areas of advanced, fast-growing interest and expanding research demonstrate the multidisciplinary nature of tissue engineering and the field of regeneration medicine. There are vast challenges as well as wide opportunities to improve human health immensely in a variety of areas. However, much study is needed to explore various novel materials and the dynamics of the bone tissue microenvironment for proper simulation.

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CHAPTER

41

Materials-Based Cancer Immunotherapies

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INTRODUCTION AND OVERVIEW OF CANCER IMMUNOTHERAPY

The immune system is composed of two primary cellular subsets; innate and adaptive. Cells of the innate immune system are often the first responders to reach a site of injury. One of their primary functions is to contain locally and eliminate, if possible, any potentially dangerous substances, which are commonly termed pathogens. In addition, a number of innate immune cells function to collect small pieces of the dangerous substance, termed antigens, and present them to cells of the adaptive immune system. Macrophages and dendritic cells (DCs) are two key cells that perform this function, and thus are appropriately known as antigen presenting cells (APCs). After successful presentation and stimulation by APCs, adaptive immune cells, namely antigen-specific cytotoxic T cells, can mount a more pathogen-specific immune response with long-term components that can protect the host more rapidly if reexposed to that particular antigen. In response to identifying features of pathogens, APCs can release specific combinations of cytokines, chemokines, and other cues to promote T-helper 1 (Th1) or T-helper 2 (Th2) adaptive immune responses. Th1 immune responses induce cell-mediated responses, typically provoking T-cell proliferation and investigation of intracellular pathogens. Alternatively, Th2 immune response induce humoral immunity, promoting B-cell antibody production targeting primarily extracellular pathogens. Although greatly simplified, this is one of the primary processes that the immune system uses any time it encounters potentially harmful substances, whether that be a virus, bacteria, or cancer cell.

Cancer presents a variety of challenges for the immune system as evidenced by the fact that avoidance of immune destruction has been recognized as a primary hallmark of cancer [1]. One of the most difficult challenges is the fact that cancer cells are not foreign entities, and thus look similar to normal healthy tissue, which makes it difficult for the immune system to identify and eliminate them. As a result, the adaptive immune system often struggles to present tumor-associated antigens in an immunogenic context that is critical for generating a productive immune response. However, cancer has long been recognized as a disease mediated by genomic mutations, whether naturally or virally induced, and if these genomic mutations result in mutated proteins, the immune system theoretically has the potential to recognize that. As proof of this, tumors with a higher mutational burden are typically more immunologically active, with higher immune cell infiltrate and a greater potential for immune-mediated attack [2]. In addition to mutated antigens, differential expression of self-antigens provides another mechanism of immunologic recognition of cancer (i.e., human estrogen receptor 2 in breast cancer, prostate-specific antigen in prostate cancer, cancer-testes antigens). Another challenge that hinders immune recognition and destruction of cancer is the highly immunosuppressive protumor microenvironment observed in solid tumors. This occurs largely through a wound healing–like intratumoral inflammation and an increase in suppressive immune cell populations such as myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), and T-regulatory cells (Tregs) [3]. In light of these obstacles, cancer immunotherapeutic strategies are warranted to direct and stimulate an effective immune response against cancer.

The idea of using one's own immune system to fight cancer is by no means a novel concept; in fact, this concept has existed for over 100 years. Perhaps the oldest recorded evidence came from an American surgeon, William B. Coley, when he demonstrated successful remission in numerous inoperable cancer patients after intratumoral

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injection of a mixture of bacterial lysates known as Coley's toxin [4]. In the 90 years after Coley's findings, the fields of immunotherapy and immunology matured in concert with one another. Significant immunotherapy achievements include the development of the first immune adjuvants [5], proposal of the immune surveillance theory or the idea that cancer cells could be recognized and killed by components of the immune system [6], proof that tumors have tumor-specific antigens that can be targeted by the immune system [7–10], and proof of tumoral immune escape through the loss of tumor-specific antigens [11]. During this same time, we note the discovery of numerous important immune mediators such as the Treg [12], DCs [13], natural killer cells [14], and interferons (IFNs), which are a family of critical protein mediators that act as signals for the immune system to regulate various aspects of an immune response [15]. Each of these discoveries and countless more were monumental in the development of the field of cancer immunotherapy.

These discoveries led to some of the first organized cancer immunotherapy clinical trials. Pioneering trials by Steve Rosenberg and colleagues, and others, aimed to use systemic administration of interleukin-2 (IL-2), a potent T cell–activating cytokine, in treating melanoma and renal cancer [16,17]. Despite promising preclinical data, clinical trials with IL-2 appeared to generate little improvement in overall survival and were often associated with concerning side effects. The early 1990s also saw the development of several promising new cancer vaccine strategies. One such strategy was GVAX, a vaccine that used irradiated cancer cells genetically modified to secrete granulocyte macrophage–colony-stimulating factor (GM-CSF), a potent APC activator [18]. A second successful strategy involved the use of autologous DCs pulsed with cancer peptides that could be reintroduced to the patient [19]. Both strategies were found to elicit potent antigen-specific T-cell responses and led to the development of Sipuleucel-T, or Provenge, for the treatment of prostate cancer. Sipuleucel-T treatment requires the isolation of patient-derived DCs and incubation with both an antigenic target overexpressed on most prostate cancers (i.e., antigenic prostatitic acid phosphatase) and GM-CSF for stimulation. Blinded, placebo-controlled phase III clinical trials showed a 4-month improvement in median survival for patients receiving Sipuleucel-T compared with placebo-treated cohorts [20]. This eventually led to the US Food and Drug Administration (FDA) approval of Sipuleucel-T for the treatment of prostate cancer in 2010. Sipuleucel-T represents the first major cancer immunotherapy to receive FDA approval and ultimately paved the way for numerous immunotherapies. A variety of other cancer vaccine strategies are currently under investigation, which vary according to what stimulus they provide and whether they deliver only a single antigen, termed subunit vaccines, or a whole library of tumor antigens (i.e., tumor lysates, irradiated whole tumor cells) [21]. During this period, adoptive T-cell therapies were also showing promise as an effective cancer immunotherapy. Applications include genetically modified T cells made to recognize and kill specific tumor antigens or chimeric antigen receptor (CAR) T-cells and adoptive T-cell therapies in which tumor reactive T-cells isolated from the patient were stimulated ex vivo and then reinfused [22–24]. Despite showing significant success in blood-borne cancers (i.e., lymphoma, leukemia), T-cell therapies continue to show minimal benefits against established solid tumor cancers; however, numerous technical advances and clinical trials are under way investigating new approaches and combinatorial treatment strategies with these therapies.

The mid 1990s saw the emergence of an immunotherapy platform termed immune checkpoint inhibitors (ICIs), which continue to show significant potential as a solid tumor therapy. Two ICI targets of extensive investigation are cytotoxic T lymphocyte–associated antigen-4 (CTLA-4) and programmed cell death protein-1 (PD-1). CTLA-4 is expressed on activated T cells and functions as the "brakes" of a T-cell response by saturating away costimulatory molecules on activated APCs and promoting overall deactivation of the T cell [25,26]. Blockade of CTLA-4 using monoclonal antibodies has been shown to prolong T-cell activation and promote significant antitumor effects in murine models [27,28]. Phase III clinical trials of Ipilimumab, a humanized antibody targeting CTLA-4, improved median survival by 4 months for patients with advanced melanoma, with a small subset of patients maintaining cancer-free progression up to 10 years later [29,30]. This led to the FDA approval of Ipilimumab in 2011 to treat advanced melanoma. CTLA-4 blockade is currently being tested in numerous other cancers both as a monotherapy and combined with other treatment modalities. Success of CTLA-4 inhibition led to the investigation of numerous other ICI targets, one of particular success being PD-1. PD-1 functions to deactivate T cells after engagement with either of its known ligands, PD-L1 or PD-L2 [25]. Of interest in cancer, high PD-L1 expression has been observed in numerous solid tumor cancers and has been correlated with a poorer prognosis [31–33]. In addition, cancer-induced exhaustion of T cells appears to upregulate PD-1 drastically on their surfaces, making them more susceptible to deactivation through this pathway [34]. Strong evidence suggests that blockade of PD-1 can restore functionality to these exhausted T cells and promote significant antitumor effects [35,36]. Thus, after clinical investigation, both nivolumab and pembrolizumab, two humanized anti-PD-1 monoclonal antibodies, received FDA approval in 2014 for the treatment of unresectable melanoma. Since then, they gained FDA approval

for the treatment of lung and kidney cancers in 2015 and Hodgkin lymphoma in 2016 and are actively being investigated in numerous other cancer types, again as both monotherapies and combined with other treatment modalities.

Despite preclinical and some clinical success of immunotherapies in treating cancer, it should be noted that although small subsets of patients have full durable responses, most patients do not benefit from these therapies. In addition, many of these therapies result in a number of systemic side effects, many of which could have life-threatening or long-term consequences for the patient's health. This leaves much to be desired from the field of immunotherapy in treating cancer.

ADVANTAGES AND DISADVANTAGES OF CANCER IMMUNOTHERAPY

Cancer immunotherapy offers numerous advantages compared with other standard of care treatments. Most notably, immunotherapy optimizes the patient's own natural immune system as the primary therapeutic source. This allows use of the intricate biologic features of the immune system that engineered therapeutic systems cannot currently achieve. Examples of these features include the natural ability of T cells to traffic and extravasate into tumor tissue, the ability to target cells preferentially harboring specific aberrations while leaving surrounding healthy tissue relatively unharmed, the ability to seek and destroy cancer cells throughout the entire body, even micrometastatic lesions, and finally, features of immunologic memory that can protect against recurrence. These advantages are highly unique to immunotherapy and thus provide the rationale for why it remains an exciting and highly investigated therapy in the fight against cancer.

Despite these numerous advantages, current immunotherapy applications face a number of challenges. Most notable is their inconsistent efficacy, because the majority of patients do not benefit from current immunotherapeutic strategies. Even more challenging is that currently few correlative biomarkers can predict patient success from these therapies. This has led numerous groups to investigate combinatorial treatment strategies in the hope of achieving synergistic treatment effects. Some examples include combinations of immunotherapies with chemotherapy, radiation, small molecule inhibitors, and even combined with other immunotherapy strategies [37]. In addition to low success rates, a number of immunotherapies have shown concerning side effects. Most often, these side effects are associated with systemic hyperactivation of the immune system, which can promote autoimmune responses against vital organ systems, leading to long-term and sometimes deadly health consequences for patients. The last major challenge of immunotherapy is affordability. Many current immunotherapies require isolation of patient-derived cells (i.e., T cells, DCs, tumors cells), which are then modified and readministered to the patient. This personalized medicine approach requires an extensive manufacturing pipeline and is often associated with vastly higher costs than a typical "off-the shelf" cancer drug such as chemotherapy or small molecule inhibitors. Non—patient derived immunotherapies are often expensive as well, typically requiring months to years of clinical visits for therapeutic administration. This creates major issues in terms of patient affordability and compliance. Thus, despite significant efforts and advances in the field of immunotherapy, it continues to remains significantly hindered by its limitations. This is where biomaterials-based approaches could have a significant role.

Whether macro-, micro-, or nanoscale, biomaterials offer the potential for sustained and/or targeted release of drugs (Fig. 41.1). For immunotherapeutic applications, this simple feature could provide a significant opportunity to overcome many of its current challenges. Biomaterials would provide a platform to contain an immune response locally within target areas. This would greatly benefit immunotherapies because the desired immunomodulation could occur in important areas such as in a subcutaneous zone, within the tumor itself, or even within critical immunologic tissues (i.e., lymph nodes and spleen). This could benefit the safety of immunotherapies by eliminating systemic immune activation and also would likely improve their efficacy, because more of the delivered drug would come into contact with the effector cell types rather than being removed from the systemic circulation or be degraded through normal physiologic routes. In addition, advances in biomaterial technology allow for accurate and highly controllable fine-tuning of drug release, in some cases providing extended release for up to years. Numerous groups have also developed systems for the controlled release of more than one drug with the potential to control the release kinetics of each drug individually. These multidrug release systems could enable the delivery of combinatorial immunotherapies to mimic the true response progression of the immune system more closely (i.e., first priming the innate system and then providing activation markers for the acquired immune system to maintain that response). This has the potential to enhance the poor response outcomes of immunotherapy greatly and could minimize the necessary time that patients need to spend in the clinical

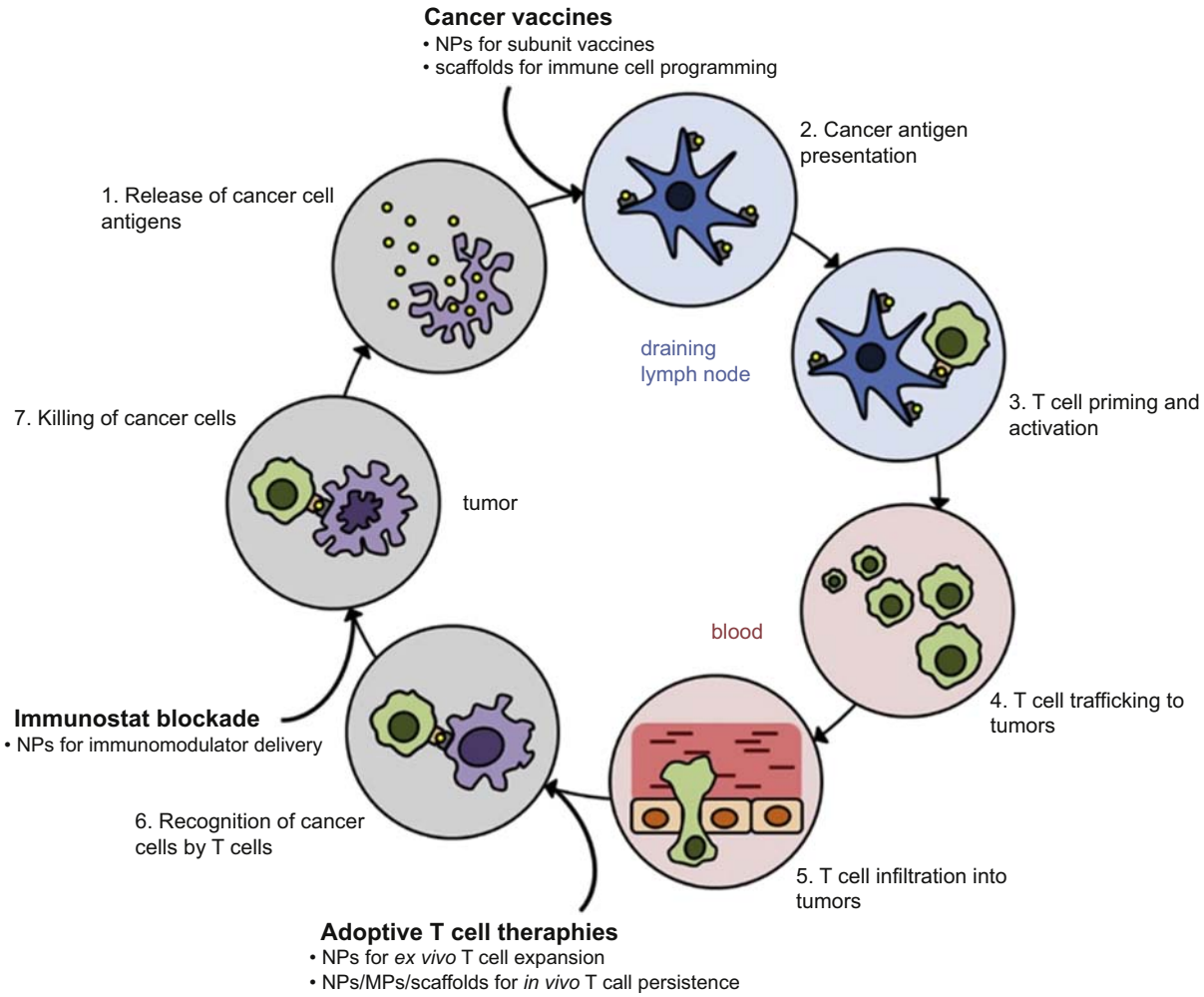

FIGURE 41.1 Schematic of the cancer-immunity cycle illustrating each sequential step with the labeled physiologic location needed to generate an efficient immune response against a tumor. Current immunotherapy approaches are shown at their point of augmentation (*bolded*) and biomaterial-based strategies used to benefit those immunotherapeutic strategies are listed below (*bulleted points*). *MPs*, microparticles; *NPs*, nanoparticles. *Reproduced with permission from Cheung AS, Mooney DJ. Engineered materials for cancer immunotherapy. Nano Today August 1, 2015; 10(4):511–31, Elsevier.*

setting, because the biomaterial could be implanted and release the drug continuously for extended times. Finally, although biomaterial approaches in immunotherapy seemingly make it more expensive, there are many opportunities for biomaterials to make these strategies cheaper. Biomaterials approaches allow the more efficient use of immunotherapeutic drugs by targeting them to key areas and cells types while minimizing physiologic clearance and drug degradation, and thus could minimize the necessary time patients need to spend in the clinic receiving the drugs. By providing a platform for immunologic modification of patient-derived cells in situ, biomaterial systems could also cut the costs of adoptive cell therapies. Thus, biomaterials have the potential to remove many of the challenges that immunotherapy currently faces.

NANOPARTICLE BIOMATERIALS FOR CANCER IMMUNOTHERAPY

Introduction of Nanomedicine in Cancer

Since its original discussion and conception in 1959 by Richard Feynman during his famous lecture, "There's Plenty of Room at the Bottom" [38], nanotechnology has continued to show exciting potential in the field of cancer therapy. The National Nanotechnology Initiative defines nanotechnology as the manipulation of matter at sizes

between 1 and 100 nm; however, many consider materials under 1 μm to warrant nanoparticle status. Because nanotechnology applications are so broad, the term "nanomedicine" further defines the application of nanotechnology in medicine. Most nanomedicine approaches rely on their intrinsic ability to accumulate in tumor tissue owing to a phenomenon known as the enhanced permeation and retention effect. Essentially, leaky tumor vasculature and inadequate lymphatic drainage result in the preferential localization of nanoparticles in the intratumoral space [39]. Thus, many groups have attempted to localize various therapeutic agents in tumors using nanoparticles to limit systemic toxicity and maximize drug efficacy [40]. In these attempts, a number of nanoparticle formulations have been widely optimized (i.e., metallic, liposome, dendrimer, and polymeric) (Fig. 41.2).

Nanoparticles offer a number of unique properties that make them an attractive approach to improving current immunotherapy applications. The biocompatibility of the formulation material, surface charge, size, and shape of the nanoparticle all have critical roles in how it interacts with the body. In particular, these features greatly influence where it localizes in the body, with what cell types it interacts or by which it is internalized, and its cytotoxicity effects [41,42]. Thus, when designing nanoparticles for a specific application, these features must be taken into careful consideration. A more detailed description of these various nanoparticle design criteria will be provided subsequently.

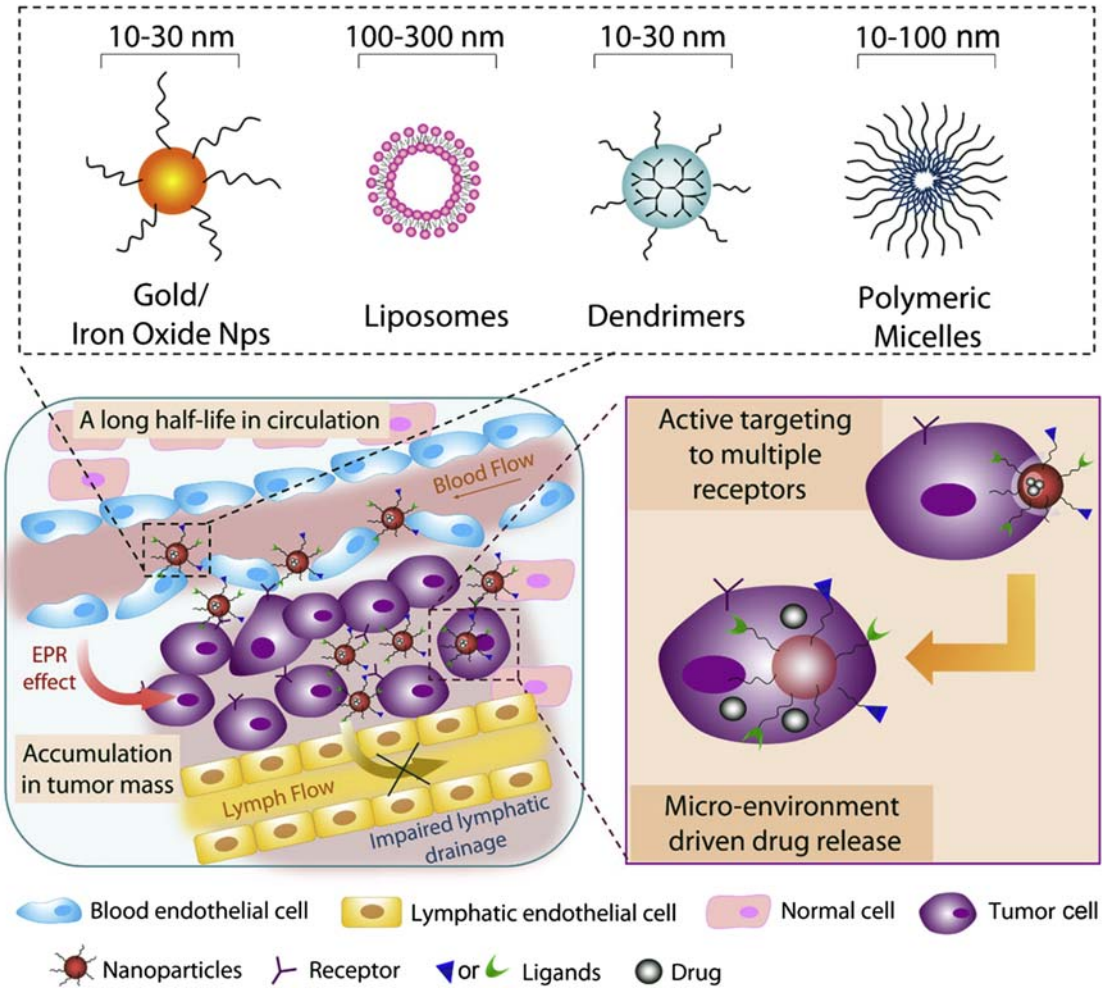

FIGURE 41.2 Schematic of the four most common nanoparticle platforms (*top*) and their application as tumor drug delivery vehicles. Optimally designed particles with adequate shape, size, surface charge, hydrophilicity, and surface modifications will promote a longer circulating half-life and accumulation in the tumor through the enhanced permeation and retention (EPR) effect (*bottom left*). Surface modification of the particle with ligands or other targeting moieties can be used to target receptor on tumor cells and promote the intracellular delivery and release of drug payloads in response to specific intracellular stimuli (*bottom right*). *Nps*, nanoparticles. *Reproduced with permission from Shao K, Singha S, Clemente-Casares X, Tsai S, Yang Y, Santamaria P. Nanoparticle-based immunotherapy for cancer. ACS Nano January 27, 2015;9(1):16—30, American Chemical Society.*

Effects of Nanoparticle Size and Shape

With improvements in nanoparticle manufacturing techniques, it is possible to make a variety of nanoparticle shapes. This has led to a number of studies determining how cellular and physiologic systems interact with different nanoparticle shapes. Cellular particle uptake rates depend on both size and shape. It appears that for particle sizes larger than 100 nm, rod-shaped particles have the highest uptake rates, followed by spheres, cylinders, and cubes. However, with particles smaller than 100 nm, sphere-shaped particles surpass rods in terms of cellular uptake rates [43]. Other more exotic shapes with various surface characteristics are being investigated; however, it remains unclear whether these will provide differential uptake.

When introducing nanoparticles to a physiologic system, several other effects such as pharmacokinetics and tissue distribution influence nanoparticle size and shape design criteria. For nanoparticles delivered intravenously, particles smaller than 6 nm are readily removed from the circulation by the kidneys. Nanoparticles larger than 200 nm typically accumulate in the liver and spleen and encounter a variety of immune cells tasked with removing large particulates from the circulation known as the mononuclear phagocytic system (MPS). Cells of the MPS typically phagocytose the nanoparticles, degrade them if possible, and then exocytose them for either filtration and removal or potential distribution in other physiologic tissues. Nanoparticle shape also is important in the physiologic systems, as evidenced by the fact that cells that circulate in the blood are typically not spherical. Thus, unsurprisingly, rod- or disk-shaped nanoparticles can promote longer circulation times compared with spherical nanoparticles [44]. Particle sizes between 10 and 200 nm are optimal in cancer nanomedicine for promoting long-term circulation, which ultimately provides more opportunities for delivery into tumor tissue. Within the tumor interstitium, particles in the upper end of this optimal range (100−200 nm) cannot extravasate far beyond the blood vessel and often remain stuck in the extracellular region between cells. Smaller particles (10 nm) have the potential to penetrate much farther into tumor tissue; however, without some form of targeting agent, they are not well-retained for more than 24 h [45]. Although many of these design optimization studies aimed for tumor drug delivery applications, in which the primary goal was to enhance nanoparticle localization within the tumor, an understanding of how particle size and shape dictate particle fate continues to shape the design characteristics of its newer applications in immunotherapy.

Effects of Nanoparticle Surface Charge and Hydrophilicity

Nanoparticle surface charge and hydrophilicity are also features that have major implications in the design of nanoparticles. These features are easily modifiable through a number of technologies such as layer-by-layer, conjugation of surface moieties, and careful selection of particle material. Generally, positively charged particles are taken up by cells at a much higher rate than are neutral or negatively charged particles. Many researchers postulate that this is the result of the slightly negative surface charge of cellular membranes, which provides an electrostatic interaction that draws positively charged particles to the surface of the cell. In addition to having higher uptake, positively charged particles induce higher cytotoxicity effects because of detrimental disturbances that they cause in cellular membranes [46]. Similar to size, however, introduction into a biologic environment creates a number of additional considerations. Most notable is that the nanoparticle surface is rapidly covered by a variety of serum proteins, forming what is known as the corona on the particle surface. The surface charge and hydrophobicity of the particle largely dictate the composition of this corona and thus influence the future fate of the particle. Most highly charged particles, positive or negative, rapidly and strongly bind a number of serum proteins that tag them for removal through the MPS system [47,48]. Particles containing a more hydrophobic surface experience a similar fate, with high serum protein binding and removal. Thus, neutral particles with highly hydrophilic surfaces are the most optimal for naturally promoting long-term circulation; however, depending on the application of the nanoparticle, this may not be the desired surface features. With these understandings, many groups have developed ways to alter the surface characteristics of their nanoparticles to provide beneficial delivery features.

Effects of Nanoparticle Surface Functionalization

As previously described, the surface characteristics of nanoparticles are a major parameter in the development and design of nanomedicine applications. Thus, numerous surface modification techniques have been developed to create favorable surface features without having to modify the bulk material of the nanoparticle. Surface attachment of hydrophilic polymers such as polyethylene glycol (i.e., PEGylation) is one of the most widely studied

nanoparticle functionalization techniques. Many groups have shown significant improvements in the particle circulation time as a result of decreased protein absorption and MPS recognition after PEGylation [49]. Another surface modification commonly used is the attachment of targeting agents. A variety of targeting moieties have been developed, the most prominent of which are variable antibody fragments, peptides, receptor ligands, and aptamers. These are often made specific to a receptor or surface target on the target cell; upon binding, they promote the internalization of the nanoparticle carrier. The small size of nanoparticles provides a vast improvement in the total surface area exposure compared with many micromaterials and macromaterials, which allows for drastic advantages for targeting applications. Numerous groups have further shown that there are a variety of parameters that must be optimized for targeting ligands to make them maximally effective; these typically rely on targeting the agent density on the nanoparticle surface and the nanoparticle size [50]. With beneficial surface characteristics, nanoparticles can be considerably improved in nanomedicine applications. These important findings have promoted many of the design criteria for applying nanoparticles in immunotherapy.

Nanoparticle Targeting Applications in Immunotherapy

Nanoparticle targeting often occurs through two primary methods, active or passive. Active targeting requires conjugating targeting moieties to the surface of the nanoparticle to encourage localization in specific areas or uptake by critical cells. Alternatively, passive targeting relies on the natural properties of the nanoparticle (i.e., surface charge, size, shape) to promote preferential uptake. Drug targeting is a unique ability of nanoparticles that has been widely studied in the field of drug delivery, with extensive success including a number of FDA-approved nanoparticle drug targeting formulations [51].

Compared with standard cancer nanomedicine targeting, immunotherapeutic applications require slightly more consideration of the intracellular or extracellular compartment in which the therapeutic payload will be released. For example, proinflammatory cytokines or other agents that target surface-bound cell receptors must be released into the extracellular tumor space. A number of strategies have been used to promote extracellular release, typically aiming to discourage phagocytosis of the particle. Notable examples include PEGylation or coating of the particle's surface with antiphagocytic signals such as CD47 [52]. In addition, fine-tuning of the particle's physical properties can be used to diminish cellular uptake, as previously discussed. In opposition to extracellular acting agents, delivery of other therapeutics such as specific Toll-like receptor (TLR) agonists, short interfering RNAs (siRNAs), or tumor-specific antigens for cancer vaccine applications require the delivered cargo to be released in specific intracellular compartments (i.e., endosome, lysosome, or cytosol). Enhancement of the particle surface with ligands targeting endocytosis-associated receptors and optimization of a particle's physical characteristics can be used to promote more enhanced cellular uptake, especially in phagocytic cells [53]. In addition to targeting key compartments, particles must be designed to release their payload within a target environment. This can be achieved by chemically modifying the particle drug carrier to release its payload upon encountering some form of location-specific stimuli. The most common release cues include low pH and specific intracellular or extracellular proteases [54]. A number of triggered release systems have also been developed in which the release stimuli is delivered exogenously (i.e., light, magnetic field, heat) and promotes particle degradation and payload release [55,56]. Certain nanoparticle systems also provide the ability to fine-tune the rate of drug release by altering various particle features such as porosity, degradability, and the drug incorporation method (basic encapsulation versus stimuli-cleavable chemical conjugation). Thus, optimizing these various features has allowed for highly efficient, selective, and fine-tunable targeting of immunotherapeutic agents. This which will be discussed in more detail subsequently.

Nanoparticle Targeting of the Tumor Microenvironment

Most solid tumor cancers harbor a wound healing–like inflammation and a highly immunosuppressive microenvironment. This feature of solid tumors is elicited by an intricate network of numerous cell types including the cancer cells themselves, a highly active stroma (i.e., cancer-associated fibroblasts), and a number of immune cells such as MDSCs, Tregs, and TAMs. Through a variety of mechanisms, this tumor microenvironment can drastically prevent effector T-cell infiltration into the tumor and abrogate the cytotoxic function of effector cells that manage to infiltrate it. The tumor microenvironment has even been shown to promote tumor growth and induce tumoral immune tolerance [3]. Thus, modulating this tumor microenvironment would provide a significant therapeutic opportunity, especially in situations in which a notable population of cancer-specific T cells exists but is unable

overcome the immunosuppressive state of the tumor. The most common immunotherapeutic applications targeting the tumor microenvironment aim to inhibit or downregulate immunosuppressive features of the tumor, stimulate suppressed effector immune cells within the tumor parenchyma, or combinations of these approaches. Bolus delivery of immunomodulatory agents (i.e., cytokines, ICIs) have rapid saturation and/or degradation, which limits the effective dose delivered to the tumor and generates significant concern for systemic inflammatory toxicities. Thus, leveraging the advantages of nanoparticles to deliver these agents selectively to the tumor is an obvious application of nanomedicine in immunotherapy.

Rather than attempt to discuss the robust amount of preclinical application of nanomedicine targeting the tumor microenvironment, we will provide a few examples of the diverse applications that are currently being explored. One interesting application involves targeting dysregulated genetic pathways within tumor-infiltrating immune cells. Signal transducer and activator transcription 3 (STAT3), a transcription factor that has previously been shown to promote Th2-based inflammation and enhance the survival of Tregs, is an intriguing target. Using tumor-targeting liposomes delivering intracellular agents to deplete STAT3 (i.e., small-molecule inhibitors [siRNA]), a few groups have shown impressive tumor immunosuppression reversal effects [57]. This strategy provides a valuable platform for the inhibition of numerous other dysregulated immunologic genetic pathways within the tumor. In addition to inhibiting immunosuppressive mechanisms directly, many groups have aimed to activate naive, suppressed, or tolerogenic effector cells within the tumor microenvironment. A number of strategies have been employed for this, including tumor localized delivery of proinflammatory and T cell−stimulating cytokines (i.e., IFN-gamma, IL-1α, IL-2, IL-12, tumor necrosis factor-α) and APC-activating agents (i.e., CD40 ligand, cytosine-guanosine oligodeoxynucleotides [CpG], lipopolysaccharide, monophosphoryl lipid A [MPL-A], polyinosinic−polycytidylic acid [poly I:C]) [58−60]. Nearly all of these nanoparticle delivery systems showed benefits compared with bolus delivery of the same agents because they provided less systemic toxicity and extended release for up to many weeks in some cases, thus promoting more potent immunomodulatory effects. These strategies have shown relatively minimal therapeutic benefits as monotherapies; however, they are readily being investigated as combination modalities with more conventional immunotherapeutic strategies. Toward this combinatorial theme, a few groups have developed dual therapeutic delivery systems encapsulated within a single nanoparticle platform. One particular group used a polymeric liposome system, termed nanolipogels, to codeliver a small molecule inhibitor for transforming growth factor-β (TGF-β) (an immunosuppressive cytokine) and a potent T cell−activating cytokine, IL-2 (Fig. 41.3A). Their system was able to extend the release of these agents for many days and promoted significant survival benefits in preclinical models (Fig. 41.3B and C) [61]. In addition to releasing proinflammatory markers, some nanoparticle systems have been shown to promote the activation of target cell types by surface engagement with effector cells. One group optimized liposomes enhanced with IL-2 fragments and costimulatory 4-1BB, which were retained at the tumor site and promoted marked T-cell activation within the tumor through active engagement [62].

Applications of nanomedicine in targeting the tumor microenvironment are extensive; the example listed earlier represents only a portion of the vast work that has been done in this area. Overall, nanoparticles provide major advantages over the bolus delivery of immunomodulatory drugs. First, they enable the agent to be localized and concentrated at the tumor site, which benefits not only its efficacy but also its systemic toxicity. Second, they allow for extended immunomodulatory drug release, which provides sufficient time for effective immune responses to be generated and mounted against the tumor. Finally, they enable the targeting of drugs not only to important cells but also to necessary spatial and temporal locations. Overall, this renders these applications highly warranted and provides the rationale for the extensive past and current investigation of these systems.

Nanoparticle Targeting of Antigen Presenting Cells

In addition to targeting the tumor directly, significant efforts have been made to target immunologic stimuli and antigenic information to key lymphoid cells or tissues using nanoparticles, primarily targeting APCs in the lymph node and spleen. DCs are commonly recognized as the most potent APCs, which makes them a logical cellular target of nanoparticle systems. By providing DCs with the appropriate “danger signals,” they have the ability to generate potent T-cell responses against antigens that they have acquired. This creates two unique applications for nanoparticles: one in which they could deliver only stimulus to DCs, in the hope that they have already acquired tumor antigens, and a second in which they could deliver both antigen and simulation to generate highly antigen-specific immune responses. Using nanoparticles, DCs can be targeted through a number of delivery routes. Because a subpopulation of DCs dwells in peripheral tissues, nanoparticles delivered into the dermal or subcutaneous space

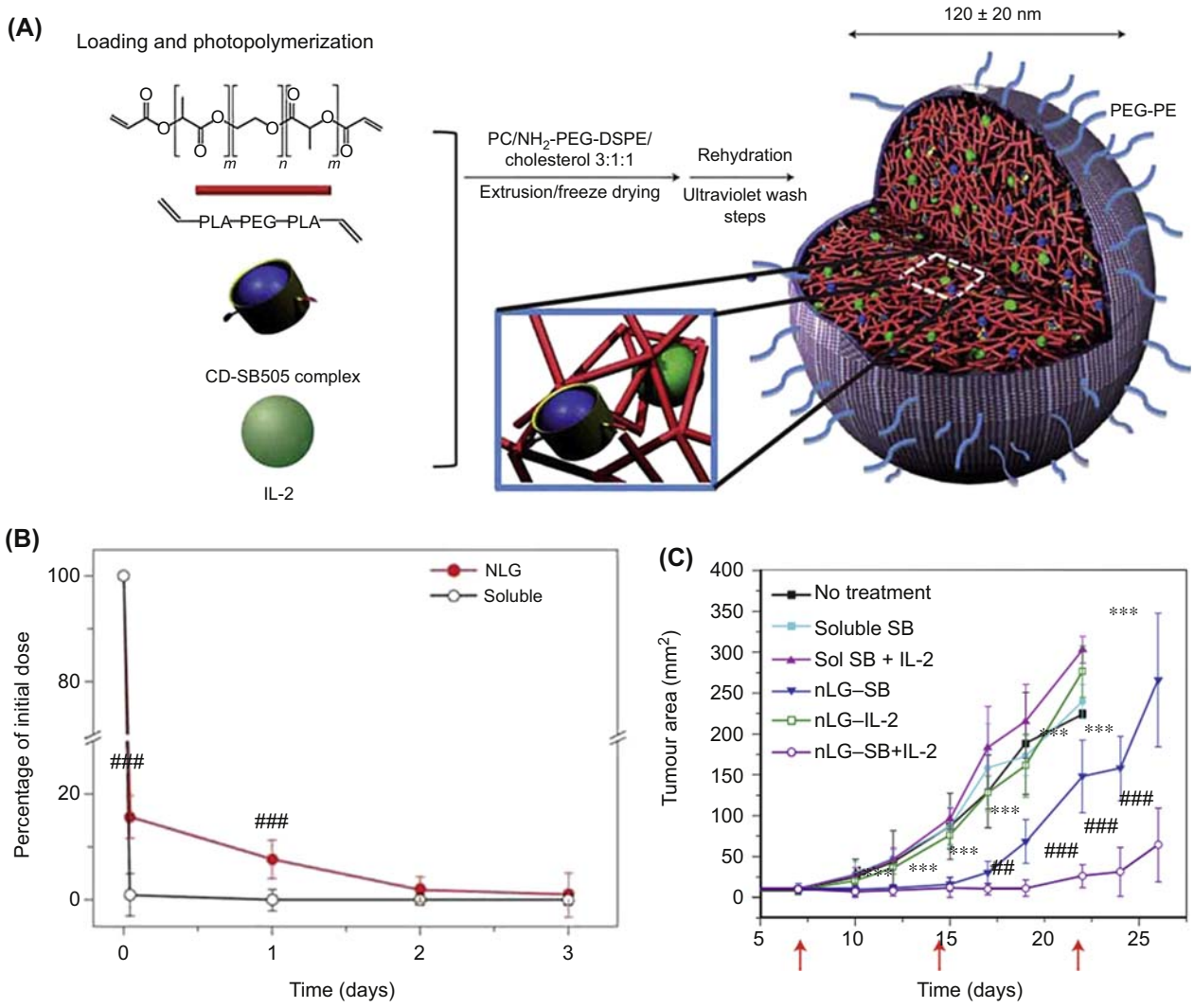

FIGURE 41.3 Nanolipogels (NLGs) are an effective immunotherapy codelivery platform (A) Synthesis illustration of NLGs. Lyophilized liposomes were loaded with a tumor growth factor-β receptor 1 inhibitor that was solubilized using methacrylate-conjugated β-cyclodextrins (SB), interleukin-2 (IL-2) cytokine, and a biodegradable cross-linking polymer. This allowed the formation of a core-shell structure with entrapped CD-SB505 (*blue*) and IL-2 (*green*) in a biodegradable polymer matrix (*red*), all within a PEGylated liposome. (B) Percentage of intravenously delivered rhodamine (a fluorescent pseudodrug) remaining at 1, 24, 48, and 72 h postinjection. Rhodamine was either encapsulated in their NLGs or bolus delivered in saline. (C) Plot of tumor area following treatment of established B16—F10 melanoma in B6 mice. Treatment groups include no treatment or intratumoral injections of soluble SB, soluble SB and IL-12, NLG-encapsulated SB, NLG-encapsulated IL-2, and NLG-encapsulated SB and IL-2. Dates of injection are indicated by *red arrows*. *PE*, polyethylene; *PEG*, poly(ethylene glycol); *PLA*, poly(lactic acid). *Reproduced with permission from Park J, Wrzesinski SH, Stern E, Look M, Criscione J, Ragheb R, et al. Combination delivery of TGF-β inhibitor and IL-2 by nanoscale liposomal polymeric gels enhances tumour immunotherapy. Nat Mater October 2012;11(10):895—905, Nature Publishing Group.*

can be phagocytosed by DCs, activate them, and promote their migration to the lymph nodes, where they can induce T cell expansion. Alternatively, nanoparticles can be designed to drain into the lymphatics and encounter lymphoid tissue—residing DCs. Both of these strategies provide a unique application for nanomedicine, because they essentially allow for DC priming and activation without the need for ex vivo cell isolation and manipulation. Thus, cancer vaccine applications using nanoparticles have promoted great excitement in the field and continue to show promising effects.

A number of groups have developed nanoparticle formulations that can be targeted to the lymph node. Lymph nodes are obviously a useful target organ because of their primary function in the priming and generation of clonal T cell expansion. Various groups have demonstrated that 20- to 100-nm particles are optimal for lymph node targeting through lymphatic drainage, with markedly faster lymph node localization and uptake at the smaller sizes [63]. At this size, particles can extravasate from vascular beds and are driven into the lymphatic network, where

they are transported to lymph nodes via interstitial pressure. Once in the lymph node, particles are readily taken up by DCs, providing an efficient route to promote immunologic priming. Various groups have then used this feature for subunit vaccine strategies, typically optimizing a model antigen ovalbumin (OVA). A study using subcutaneously injected PEGylated lipid nanoparticles with encapsulated OVA and cyclic dinucleotide stimulus promoted significant lymph nodal immune responses. Using their system, they noted a 15-fold improvement in adjuvant localization to the lymph node and a threefold improvement in OVA-specific CD8+ T-cell generation compared with bolus antigen and adjuvant delivery [64]. To improve on poor liposome serum stability, another group developed multilayered liposomes with intralayer cross-linking, termed interbilayer-crosslinked multilamellar vesicles (ICMVs). PEGylated ICMVs with OVA loaded into the particle center and MPL-A, a TLR-4 agonist, embedded within the ICMV wall promoted a 14-fold enhancement in antigen-specific T cells compared with bolus delivery [65]. Another group developed an alternative strategy using the natural trafficking phenomenon of serum albumin to traffic nanoparticles to the lymph node efficiently and selectively. Their most successful lipid formulation was composed of a lipophilic albumin-binding tail, a polar linking chain, and either a potent APC agonist or a target antigen peptide (Fig. 41.4A) [66]. When delivered together, this adjuvant and antigen nanoparticle formulation complexed with albumin and was highly localized to the lymph nodes, where they promoted significant activation and expansion of target antigen-specific CD8+ T cells (Fig. 41.4B and C). Impressively, when delivered in combination with a tumor-targeting antibody, modified IL-2 cytokine, and ICI therapies, the lymph node−targeting nanoparticles were also able to promote the rejection of large established tumors (Fig. 41.4D) [67]. Overall, these subunit vaccine nanoparticle strategies provide a promising method for generating antitumor T-cell responses.

Despite the relative success of subunit vaccine strategies, they have a number of limitations. Most notable is that targeted vaccine strategies against a single antigen will not benefit cancers with unknown or unpredictable antigenic features. In addition, these strategies do not consider the vast patient-specific tumor antigen repertoire, which could ultimately promote immunoediting (i.e., selection of only the minimally immunogenic cancer cell populations) and eventual immunologic escape by the tumor. This has driven a number of groups to investigate the delivery of adjuvant nanoparticles to the tumor-draining lymph nodes (tdLNs). The tdLNs are an attractive target for simulation because they are continuously exposed to a plethora of cancer-specific antigens throughout the course of tumor progression. However, they also represent a relatively challenging stimulation site, with depleted effector cell populations and high levels of cellular suppression and exhaustion, especially at later stages of tumor progression [68]. Nevertheless, numerous nanomedicine attempts have been made to stimulate tdLNs, most often involving the delivery of APC stimulatory agonists in the hope of promoting clonal T cell expansion targeting numerous cancer antigens. One example includes the use of a 30-nm pyridyl disulfide (PDS) nanoparticle surface conjugated with CpG. The CpG conjugation was performed using pH-sensitive chemistry to promote release in the endolysosomal compartment of DCs for TLR-9 stimulation. PDS−CpG nanoparticles delivered intradermally near the tumor (B16−F10 melanoma) allowed for efficient draining and uptake by DCs in the ipsilateral lymph node, which the researchers verified to be tdLN by intratumoral injection of fluorescent dextran. These stimulatory particles increased the percentages of activated DCs and promoted a marked increase in CD8+ T cells specific for tyrosinase-related protein-2 (TRP2), a tumor-specific antigen known to be associated with this tumor model; they also significantly slowed tumor growth [69].

Although targeting of lymph nodes using an intradermal or subcutaneous injection of nanoparticles has shown promising immunologic effects, this approach raises a number of questions when considering applying them in the clinical setting. The most obvious question is which specific lymph nodes these systems should target, because the tdLN may contain the most tumor antigen−experienced DCs, however, it also contains the highest levels of immunosuppression and exhaustion, which could hinder effective stimulation. This issue spurred the development of a systemically delivered nanoparticle vaccine strategy that could be delivered intravenously and would naturally accumulate in a variety of lymphoid tissues such as spleen, lymph nodes, and bone marrow. Using systemically delivered RNA liposome complex (RNA-LPX) nanoparticles, one group demonstrated an impressive ability to target lymphoid tissues and resident DCs and macrophages passively simply by altering the net surface charge of their particles (Fig. 41.5A and B). Even more impressive was the fact that systemic delivery of their optimally charged RNA-LPX could promote significant activation of DCs and strong expansion of antigen-specific T cells (Fig. 41.5C). This ultimately promoted considerable rejection and survival benefits in a variety of established murine tumor models (Fig. 41.5D) [70].

The application of nanoparticles as APC targeting agents is one of the most exciting developments in the field of nanomedicine. Numerous groups have shown the potent effects that APC-targeted nanoparticles can induce, especially in terms of generating tumor-specific T-cell expansion. The unique ability of small nanoparticles to traffic to

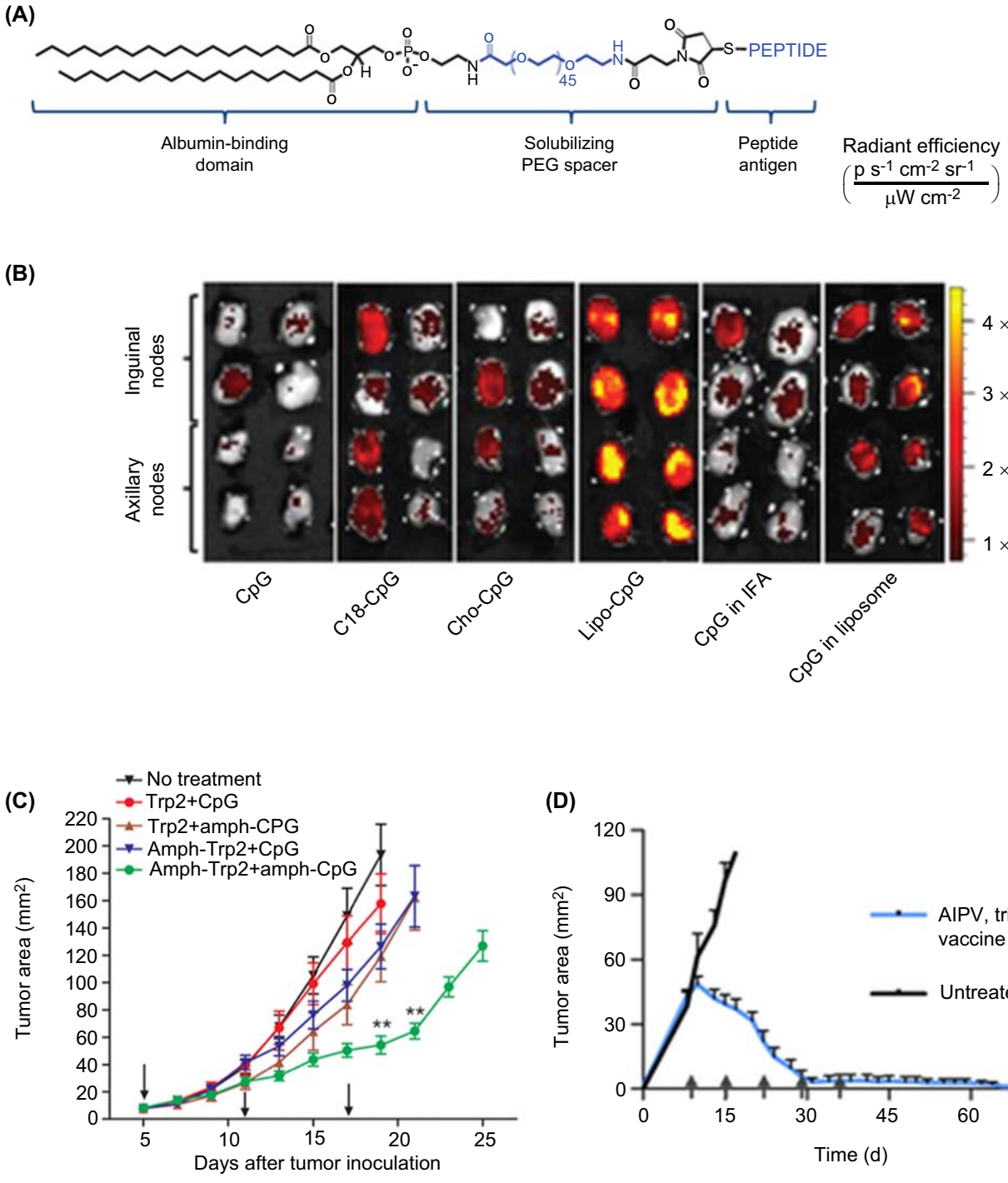

FIGURE 41.4 Amphiphilic peptide and adjuvants efficiently target lymph nodes and promote antigen-specific immune responses. (A) Structure of amphiphilic peptide used to complex with serum albumin and target peptide antigens the lymph nodes. For treatment applications, this was codelivered with liposome cytosine-guanosine oligodeoxynucleotides (CpG). (B) IVIS fluorescence imaging showing both axillary and inguinal lymph node accumulation of various targeted molecular adjuvant formulations. CpG was conjugated with fluorescein amidite to make it fluorescent. Higher intensity indicates higher CpG localization to the lymph node. Groups tested include free CpG, CpG in incomplete Freund's adjuvant (CpG in IFA), CpG encapsulated in a poly(ethylene glycol) (PEG)-coated liposome (CpG in liposome), or various amphiphile CpG conjugates; mono-acyl-conjugated CpG (C18-CpG), cholesterol-conjugated CpG (Cho-CpG), and diacyl lipid-conjugated CpG (lipo-CpG). Of note, lipo-CpG was found to associate strongly with albumin in serum (not shown), a likely mechanism of this delivery. (C) Plot of tumor area after vaccination treatment of established B16−F10 melanoma tumors in C57/BL6 mice. Mice were vaccinated with CpG and Trp2 peptide, an antigen known to be associated with B16−F10 tumors in bolus form or in designed amphiphile (amph) forms at the days indicated by *black arrows*. (D) Similar tumor area curves in B16−F10 melanoma tumors. AIPV trivalent-treated mice received vaccination with amphiphilic CpG and peptide antigens (Trp1, Trp2, and gp100) known to be associated with this tumor model, systemic administration of tumor targeting antibodies (targeting Trp1), systemic administration of albumin-fused interleukin-2, and administration of α-programmed cell death protein-1 immune checkpoint inhibitor. *(A−C) Reproduced with permission from Liu H, Moynihan KD, Zheng Y, Szeto GL, Li AV, Huang B, et al. Structure-based programming of lymph-node targeting in molecular vaccines. Nature. March 27, 2014;507(7493):519−22, Nature Publishing Group. (D) Reproduced with permission from Moynihan KD, Opel CF, Szeto GL, Tzeng A, Zhu EF, Engreitz JM, et al. Eradication of large established tumors in mice by combination immunotherapy that engages innate and adaptive immune responses. Nat Med October 24, 2016;22(12):1402−10, Nature Publishing Group.*

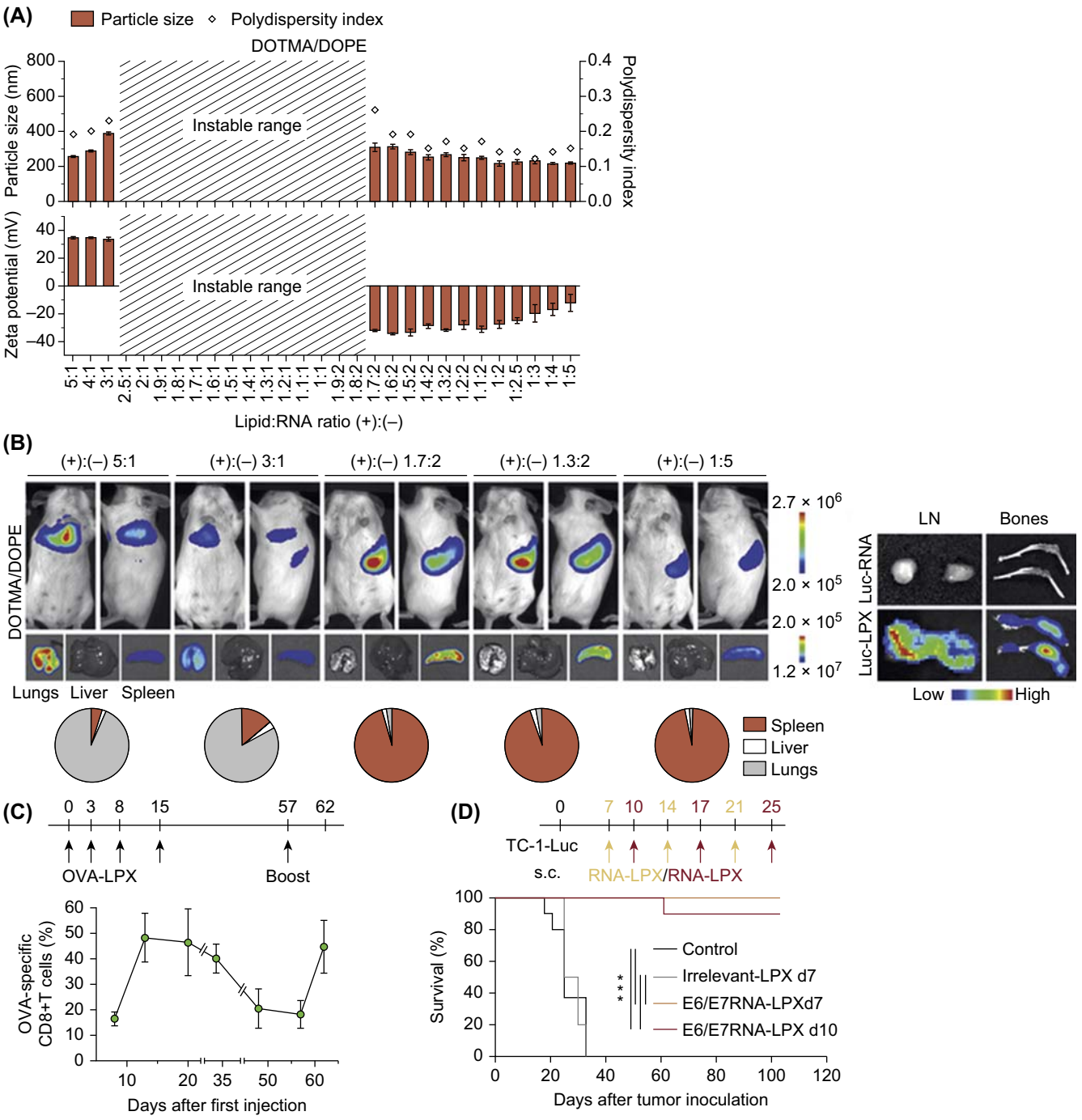

FIGURE 41.5 RNA-lipoplex (RNA-LPX) lipid carriers promote lymphoid tissue targeting potent antigen-specific immune responses. (A) Particle size/polydispersity index (*top*) and zeta potential (*bottom*) for RNA-LPX achieved by using different lipid (DOTMA/DOPE) to RNA ratios. (B) Bioluminescent imaging of Balb/c mice after intravenous injection of various luciferase encoding lipid−RNA ratios. A representative fluorescent image of a lung, liver, and spleen from each ratio group is shown below whole-mouse images with pie charts summarizing the distribution of signal in each. Fluorescent image to the far right shows additional uptake of RNA-LPX in lymph node and bone marrow at a 1.3:2 charge ratio. (C) Kinetics of ovalbumin (OVA) specific CD8+ T cells in the blood of C57/BL6 mice after multiple intravenous injections of OVA-encoding LPX carriers. Injection schedule is noted above the figure with *black arrows* indicating dates of OVA-LPX administration. (D) Kaplan−Meier survival curves of C57/BL6 mice with established TC-1 tumors harboring E6 and E7 human papillomavirus oncoproteins as target antigens. Treatment groups include untreated (control) or intravenous administration of irrelevant OVA-LPX at day 7 (Irrelevant-LPX d7), E6/E7-LPX at day 7 (E6/E7RNA-LPXd7), or E6/E7-LPX administered at day 10 (E6/E7RNA-LPX d10). The treatment schedule can be seen above the figure, with *yellow arrows* indicating the administration dates for groups started on day 7 and *red arrows* for the group started on day 10. *Reproduced with permission from Kranz LM, Diken M, Haas H, Kreiter S, Loquai C, Reuter KC, et al. Systemic RNA delivery to dendritic cells exploits antiviral defence for cancer immunotherapy. Nature 2016;534(7607):396−401, Nature Publishing Group.*

lymphoid tissues and readily be internalized by APCs, namely DCs, provides a unique application of nanomedicine in treating cancer. Furthermore, the improved efficacy and safety that nanoparticle targeted systems provide compared with the bolus delivery of immune stimulatory agents and/or antigens is unparalleled. Thus, many of these systems have translated to the clinical setting and likely will continue to do so, where it is hoped that they will provide immunotherapy applications with these pronounced benefits.

Conclusion of Nanomedicine Applications in Immunotherapy

Unsurprisingly, nanomedicine applications have demonstrated amazing potential for the enhancement of cancer immunotherapies. This section focused largely on their targeting applications, such as targeting of the tumor microenvironment or targeting of APCs in important lymphoid sites. However, nanomedicine is being applied in many other applications in immunotherapy as well [71–73]. Most of these applications apply the extended release features of nanoparticle systems to the delivery of immunomodulatory agents. One such example involves the ex vivo conjugation or the in vivo targeting of T cells using nanoparticles that provide the extended release of T-cell stimuli or inhibitors of T-cell inactivation. These "T-cell piggyback" nanoparticle systems have shown amazing potential for prolonging the persistence of adoptively transferred T-cell therapies, a major hindrance for those therapies [74,75]. This demonstrates just one example of how nanoimmunosystems could theoretically be applied to target any immune cell population and deliver any number of immunomodulatory agents, which thus providing much future work for the field of nanomedicine immunotherapy. Overall, the applications of nanomedicine in immunotherapy will likely continue to exploit the unique ability of nanoparticles to prolong the release kinetics and selectively deliver immunotherapeutic agents to important cell types or physiologic locations. This simplistic-seeming ability has the potential to benefit immunotherapeutic strategies greatly in cancer, making them safer, more efficacious, and applicable to far more cancer settings than was previously thought achievable.

MACROSCALE BIOMATERIAL SCAFFOLDS FOR CANCER IMMUNOTHERAPY

Implantable Biomaterial Scaffolds as Cancer Vaccines

As we have discussed, the ability of nanoparticles to target bioactive factors toward important cell populations at specific physiologic locations is an example of how biomaterials have evolved over the past decades to be more than just inert, biologically compatible replacement parts. This paradigm shift toward the design of "smart," biologically active materials that can manipulate and interact with cells in their environment is a fundamental concept familiar to those in the field of tissue engineering and regenerative medicine. For example, macroscale drug delivery biomaterial platforms (i.e., with at least one dimension large than 1 mm) are widely used to control the spatiotemporal delivery of multiple bioactive molecules and/or cells to direct cell behavior and drive functional tissue formation [76]. The ability of these engineered scaffolds to perform in situ cell programming has made them an attractive technology to improve DC and T cell function in the context of therapeutic cancer vaccines.

In contrast to nanoparticulate vaccines, which target antigen and/or adjuvant components to DCs located in tumor-draining lymph nodes, the paradigm for using microscale and macroscale cancer vaccines is to recruit large numbers of immature DCs to the scaffold itself, where in situ programming takes place in a controlled microenvironment. This has the potential to reduce the financial and regulatory burdens associated with conventional methods of DC-based cancer vaccinations such as Sipuleucel-T, which require cell isolation, ex vivo cell culture and manipulation, and multiple patient procedures [77]. With the in situ DC vaccination approach, the implanted biomaterial scaffold is designed for controlled release of a recruitment factor that promotes the trafficking of immune cells to the implantation site. Once there, recruited cells such as immature DCs infiltrate the scaffold and are simultaneously presented with tumor antigen and proinflammatory "danger signals" in the form of a pattern recognition receptor ligand adjuvant. The mature, antigen-loaded DCs that are generated then traffic out of the scaffold toward draining lymph nodes, where they can facilitate anticancer immunity through T cell priming and activation (Fig. 41.6).

This approach was first described by Ali et al. [77], who used macroporous scaffolds composed of the copolymer poly(lactide-*co*-glycolide) (PLG) as subcutaneously implanted DC cancer vaccines (Fig. 41.7).

Incorporated into the PLG matrix were GM-CSF as a recruitment factor for immune cells, cationic nanoparticles of CpG as the danger signal, and melanoma tumor lysate as the antigen. Sustained release of bioactive GM-CSF was

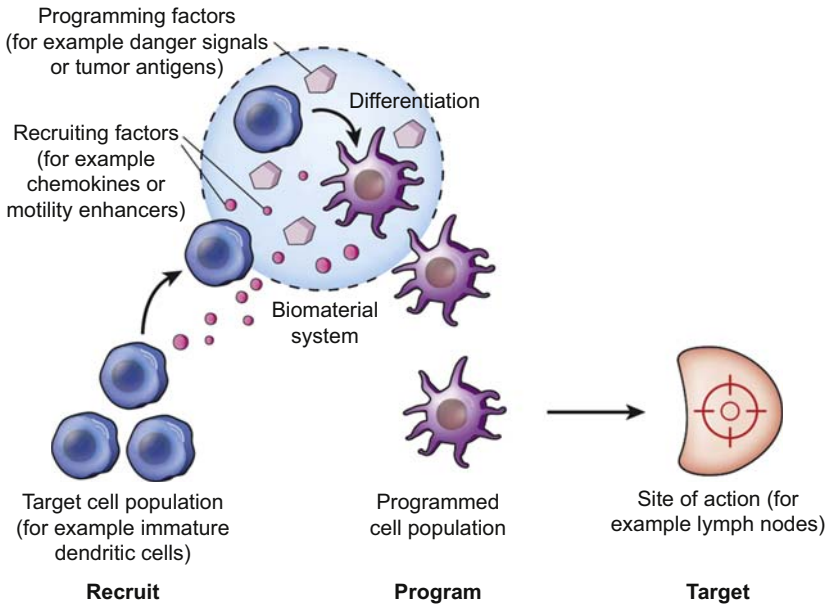

FIGURE 41.6 Schematic of an implantable biomaterial system that can be used for in situ dendritic cell programming. Recruitment factors released from the implant promote the trafficking of immature dendritic cells to the scaffold, where they encounter programming factors such as adjuvant and tumor antigen. The activated, antigen-presenting dendritic cells then leave the scaffold and migrate to draining lymph nodes, where they trigger downstream events such as the activation of antigen-specific cytotoxic lymphocytes. *Reproduced with permission from Huebsch N, Mooney DJ. Inspiration and application in the evolution of biomaterials. Nature November 26, 2009;462(7272):426–432, Nature Publishing Group.*

achieved over 15 days in vivo, resulting in a significant number of DCs infiltrating the scaffold (on the order of 10^6 cells, similar to the number of cells administered by ex vivo protocols). Scaffold-infiltrating DCs were shown to be activated by the CpG immobilized within the scaffolds and then subsequently dispersed to draining lymph nodes. After vaccination, cytotoxic lymphocytes targeting the melanoma-associated antigen TRP2 were markedly expanded in the spleen, and a threefold increase in CD8+ T-cell infiltration into tumors was noted for vaccinated mice compared with controls. Further work to characterize the multiple DC subtypes recruited to the implanted vaccine revealed the accumulation of a heterogeneous DC network including CD8+ DCs (important for cross-presentation of tumor antigen to Th1 T cells and cytotoxic lymphocytes) and plasmacytoid DCs (known to generate

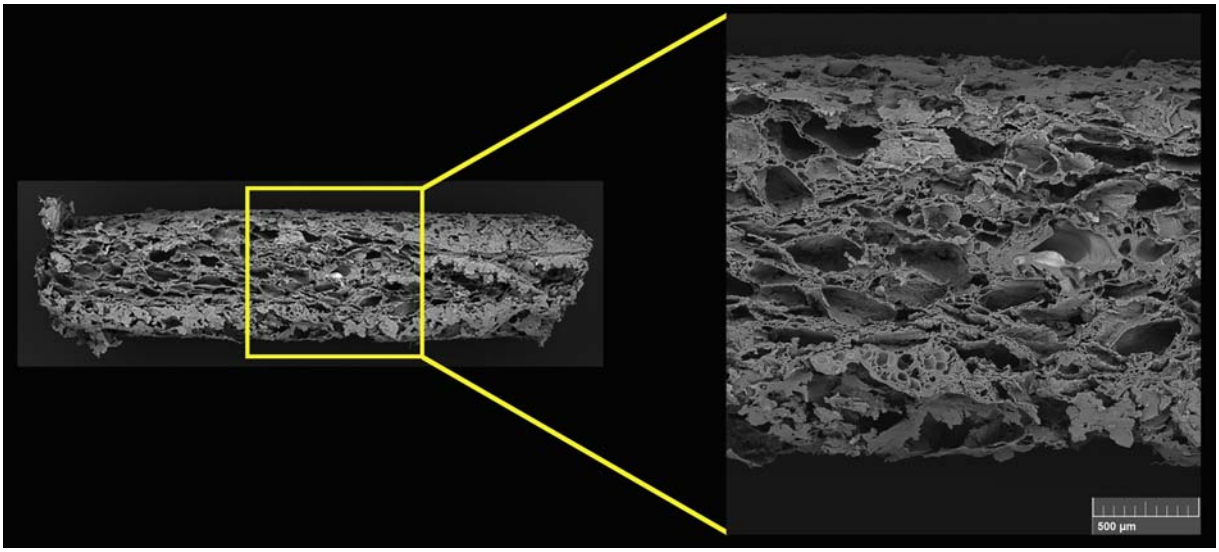

FIGURE 41.7 Low- and high-power scanning electron microscopy cross-sectional images of a macroporous poly(lactide-*co*-glycolide) cancer vaccine scaffold containing granulocyte macrophage–colony-stimulating factor, cytosine-guanosine oligodeoxynucleotides, and tumor antigen. The image on the left (low power) is 1X and the image on the right (high-power) is 125X.

type I IFNs, helping to activate CD8+ DCs) [78]. Prophylactic vaccination resulted in a 90% survival rate, whereas mice bearing established melanoma tumors and treated twice with the PLG vaccine resulted in 47% survival (Fig. 41.8). Subsequent work investigated the use of alternative adjuvants in the PLG vaccine including MPL-A and poly I:C [79], and alternative recruitment factors such as chemokine (C−C motif) ligand 20 (CCL20) and Fms-related tyrosine kinase 3 ligand [80], which illustrates the ability of this implantable, polymeric vaccine system for use as a versatile macroscale biomaterial platform to deliver multiple immunomodulatory factors. The PLG-based cancer vaccine is undergoing a phase I clinical trial under the name "WDVAX" (NCT01753089), investigating its safety in metastatic melanoma patients.

Injectable Biomaterial Systems as Cancer Vaccines

Drawbacks of macroporous scaffolds composed of PLG are their relative stiffness and brittleness, which necessitate a small surgical procedure to enable subcutaneous implantation. To address this shortcoming, macroporous Cryogel scaffolds have been developed that can undergo large amounts of reversible deformation and rapid volumetric recovery, enabling the minimally invasive delivery of these scaffolds through small-bore needles [81]. Bencherif et al. [81] investigated an alginate-based Cryogel system as an injectable cancer vaccine scaffold, incorporating GM-CSF as the DC recruitment factor, CpG as an adjuvant, and irradiated B16−F10 melanoma cells as the antigen source (Fig. 41.9) [82]. To enhance the retention of the irradiated cells within the pores of the sponge-like vaccine scaffold, arginylglyclaspartic acid (RGD) peptides were covalently coupled to the alginate, allowing the preloaded cancer cells to adhere via integrin binding and remain localized to the scaffold microenvironment. Akin to the strategy used by the PLG-based cancer vaccine, this macroporous Cryogel-based cancer vaccine was designed to provide a localized immunogenic niche for recruited DCs and irradiated tumor cells to interact in the presence of the CpG danger signal without the tolerogenic factors present in the tumor microenvironment. Successful encapsulation of GM-CSF and CpG into the sponge-like alginate cancer vaccine construct was followed by sustained release of these factors over 1 month, enabling the recruitment and activation of both CD8+ and plasmacytoid DCs and a strong effector T cell response. Mice bearing B16−F10 melanoma tumors and treated with the Cryogel vaccine at days 3 and 10 after tumor inoculation displayed a 40% survival rate compared with unimmunized mice, illustrating the potent antitumor response that can be generated with this system.

Another injectable, materials-based cancer vaccine platform was described by Kim et al. [83] and is based on high−aspect ratio mesoporous silica rods (MSRs). Using the same paradigm of host immune cell recruitment and

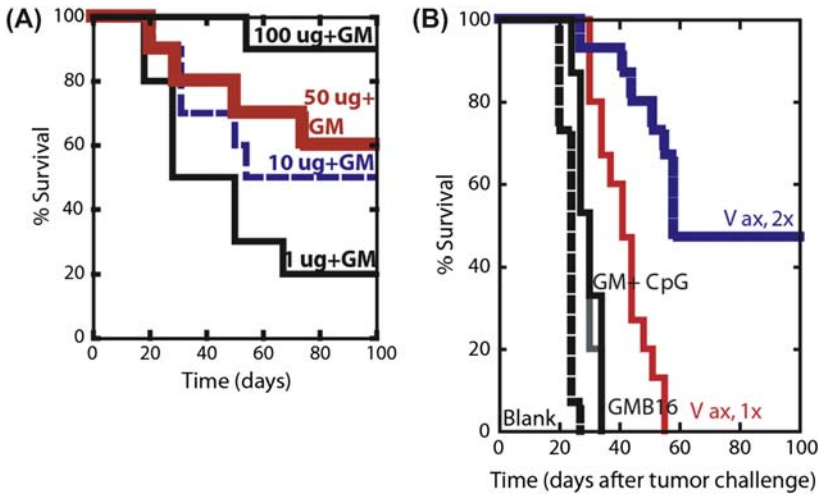

FIGURE 41.8 Kaplan−Meier survival curves of mice vaccinated with poly(lactide-*co*-glycolide) (PLG) vaccines. (A) PLG vaccine implanted 14 days before B16−F10 melanoma tumor challenge, comparing groups receiving either blank PLG scaffold or PLG scaffolds loaded with tumor lysate, granulocyte macrophage (GM)−colony-stimulating factor (GM-CSF), and 1, 10, 50, or 100 μg of cytosine-guanosine oligodeoxynucleotides (CpG). Mice receiving vaccines with 100 μg of CpG had a survival rate of 90%. (B) PLG vaccine implanted 9 days after B16−F10 melanoma tumor challenge, comparing groups receiving either blank PLG scaffold or PLG scaffolds loaded with tumor lysate, GM-CSF, and 100 μg CpG administered either once (on day 9, labeled Vax, 1×) or twice (days 9 and 19, labeled Vax, 2×). *Reproduced with permission from Ali OA, Emerich D, Dranoff G, Mooney DJ. In situ regulation of DC subsets and T cells mediates tumor regression in mice. Sci Transl Med. November 25, 2009;1(8):8ra19, The American Association for the Advancement of Science.*

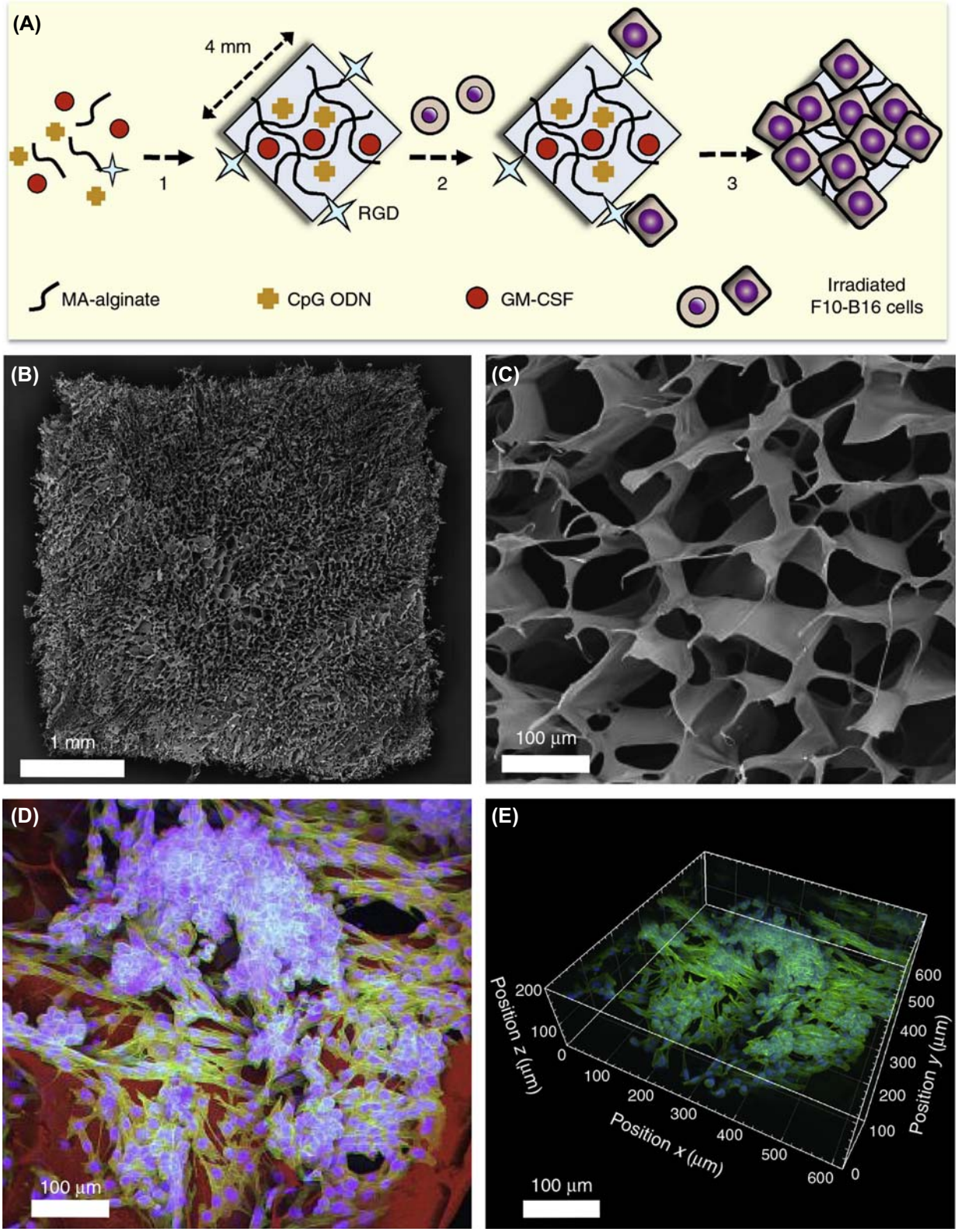

FIGURE 41.9 (A) Schematic showing how alginate-derived Cryogel cancer vaccines are prepared. Cytosine-guanosine oligodeoxynucleotides (CpG), granulocyte macrophage–colony-stimulating factor (GM-CSF), and arginylglyclaspartic acid (RGD)-containing methacrylated-alginate undergo cryogelation at subzero temperatures. Gels are then seeded with irradiated melanoma cells and incubated for 6 h before subcutaneous injection. (B) Scanning electron microscopic (SEM) image of the macroporous Cryogel. (C) Cross-sectional SEM image of macroporous Cryogel showing interconnected pores. (D) 2D confocal micrograph showing seeded melanoma cells on the Cryogel. Intracellular actin is shown in *green*, cell nuclei in *blue*, and Cryogel polymer walls in *red*. (E) 3D reconstruction of confocal image showing spreading and elongation of seeded irradiated melanoma cells after 6 h in culture. *ODN*, oligodeoxynucleotides. *Reproduced with permission from Bencherif SA, Warren Sands R, Ali OA, Li WA, Lewin SA, Braschler TM, et al. Injectable cryogel-based whole-cell cancer vaccines. Nat Commun August 12, 2015;6(May):7556, Nature Publishing Group.*

modulation in vivo, a suspension of MSRs loaded with GM-CSF, CpG, and antigen was injected subcutaneously, leading to spontaneous nonspecific self-assembly in situ to form a 3D macroporous structure akin to "thrown matchsticks" (Fig. 41.10) [83].

The high pore volume and large surface area associated with the MSRs allowed for the continuous release of bioactive GM-CSF and CpG over several weeks in vitro and promoted the persistence of a model antigen (OVA) at the vaccination site compared with bolus control by an order of magnitude (Fig. 41.11) [83]. The MSR vaccine generated a potent adaptive immune response, not only increasing the frequency of antigen-specific cytotoxic lymphocytes but also eliciting strong and durable titers of antigen-specific serum immunoglobulin G_{2a} (IgG_{2a}) antibodies associated with a Th1 inflammatory immune response. By comparison, the conventional adjuvant alum combined with the model antigen ovalbumin was able to elicit only serum anti-ovalbumin IgG_1 antibodies, skewing toward a Th2 more tolerogenic immune response. A single injection with the MSR-based cancer vaccine in the prophylactic setting was able to reduce tumor growth rate significantly and enhance survival over unvaccinated controls in a murine model of lymphoma; this illustrates the potential utility of MSRs for use as an injectable biomaterial scaffold in cancer immunotherapy.

Implantable Biomaterial Scaffolds to Enhance Autologous T Cell Therapy

Macroscale biomaterials have also been used to augment other steps in the cancer-immunity cycle [84]. Whereas the work of Ali and coworkers focused on designing systems for the in situ programming of DCs in the context of cancer vaccines, others used a biomaterials approach to address downstream events in the cancer-immunity cycle. Adoptive T cell therapy, for example, involves the infusion of autologous tumor-reactive lymphocytes to target malignancies. Maintaining the presence and antitumor potency of the bolus-injected T cells has been challenging, however. Furthermore, infused lymphocytes have difficulty tracking to the site of the tumor and overcoming the immunosuppressive tumor microenvironment when they reach it, which results in limited efficacy of this modality against solid malignancies [85]. To improve the in vivo expansion and potency of lymphocytes used for adoptive cell therapy, Stephan et al. [86] described the use of a macroporous alginate scaffold to deliver T cells to accessible or resected tumors. The scaffolds were covalently modified with a lymphocyte adhesion peptide and stimulatory cues were presented to the loaded T cells by incorporating lipid-coated silica microparticles containing the soluble factor IL-15 superagonist and bound to costimulatory anti-CD3, anti-CD28, and anti-CD137 antibodies (Fig. 41.12) [86].

Adhesion peptide-modified scaffolds increased the transit of loaded lymphocytes by 6.3-fold in vitro versus unmodified scaffolds whereas the addition of the stimulatory silica microparticles boosted in vitro T cell proliferation by 22-fold. In a mouse 4T1 breast tumor resection model, tumor-reactive T cells were delivered to the resection

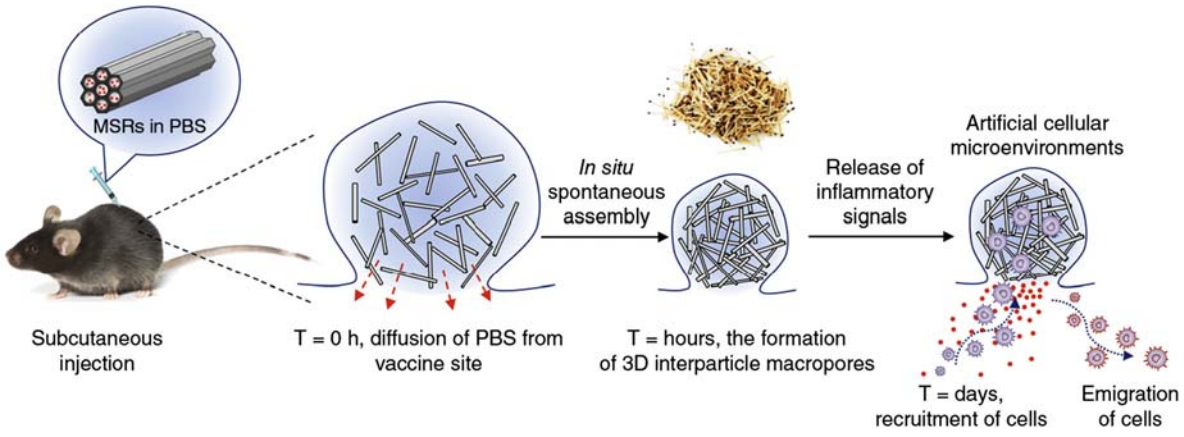

FIGURE 41.10 Schematic of mesoporous silica rod (MSR)-based vaccine in situ self-assembly and mechanism of action. MSRs dispersed in phosphate-buffered saline (PBS) are injected subcutaneously, forming a pocket. As PBS diffuses away from the site of injection, MSRs randomly and spontaneously self-assemble into a macroporous structure, allowing for host immune cell recruitment (via release of recruitment factors from the MSRs), programming (via antigen and danger signals incorporated into the vaccine), and finally emigration of programmed cells to target structures such as draining lymph nodes. *Reproduced with permission from Kim J, Li WA, Choi Y, Lewin SA, Verbeke CS, Dranoff G, et al. Injectable, spontaneously assembling, inorganic scaffolds modulate immune cells in vivo and increase vaccine efficacy. Nat Biotechnol January 2015;33(1):64—72, Nature Publishing Group.*

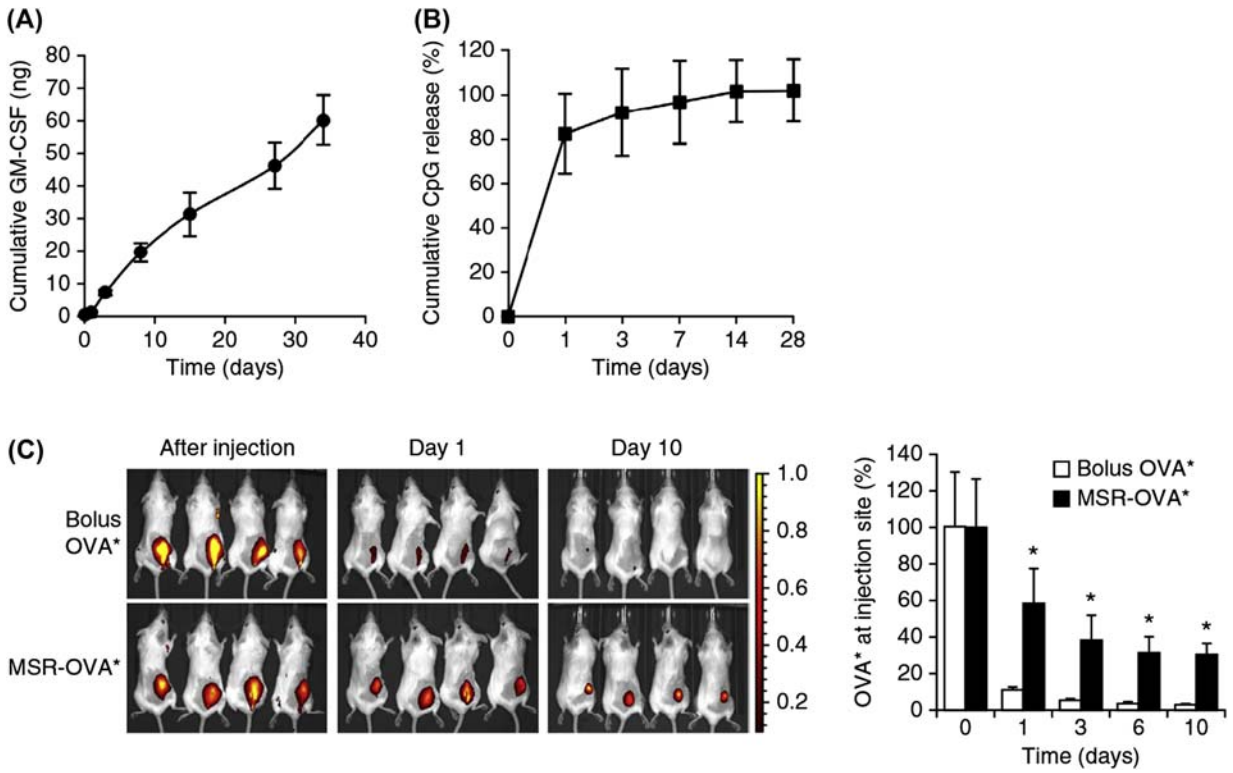

FIGURE 41.11 Recruitment factor (granulocyte macrophage–colony-stimulating factor [GM-CSF]) and danger signal (cytosine-guanosine oligodeoxynucleotides [CpG]) are released from mesoporous silica rod (MSR) scaffolds in a sustained manner. (A) In vitro release curve of bioactive GM-CSF from MSRs. (B) In vitro release curve of CpG from MSRs. (C) MSRs allow for enhanced persistence of a model antigen at the vaccination site. *Left panel* shows near-infrared fluorescent images of mice injected with a bolus of fluorescently labeled ovalbumin (OVA*) versus MSRs loaded with the same antigen; *right graph* shows relative OVA remaining at injection sites over time. *Reproduced with permission from Kim J, Li WA, Choi Y, Lewin SA, Verbeke CS, Dranoff G, et al. Injectable, spontaneously assembling, inorganic scaffolds modulate immune cells in vivo and increase vaccine efficacy. Nat Biotechnol. January 2015;33(1):64–72, Nature Publishing Group.*

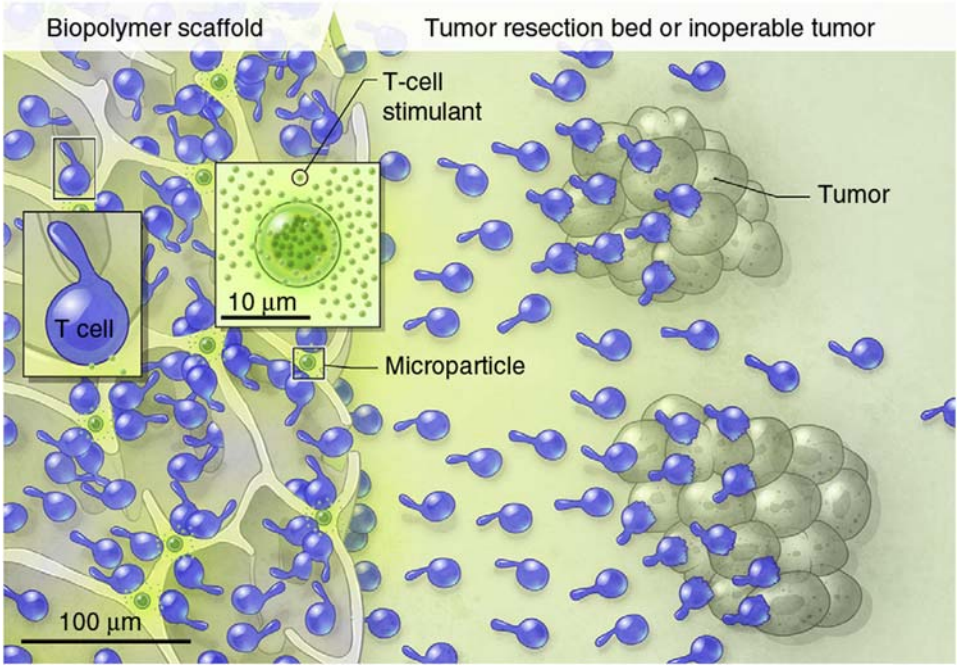

FIGURE 41.12 Schematic of a macroporous alginate scaffold used to deliver antitumor T cells (*blue*) to the tumor site via surgical placement. Stimulatory silica microparticles (*green*) contained in the scaffold promote T-cell activation and expansion, followed by migration of the activated immune cells into the surrounding environment. *Reproduced with permission from Stephan SB, Taber AM, Jileaeva I, Pegues EP, Sentman CL, Stephan MT. Biopolymer implants enhance the efficacy of adoptive T-cell therapy. Nat Biotechnol January 2015;33(1):97–101, Nature Publishing Group.*

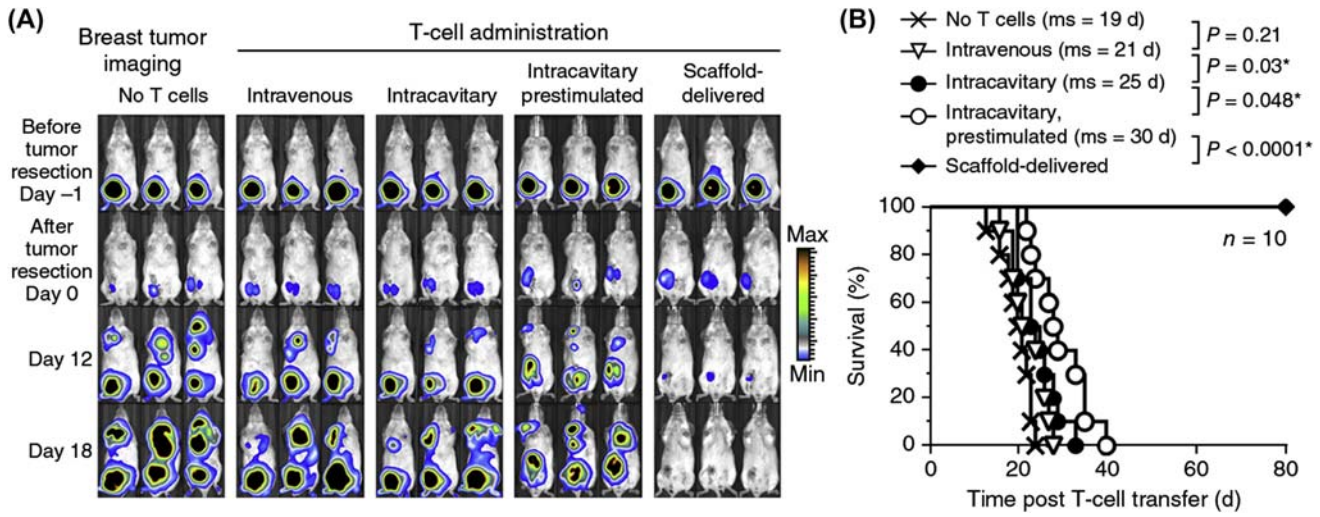

FIGURE 41.13 Tumor-reactive T cells delivered to the tumor site using the macroporous alginate scaffold−stimulatory microparticle system can eliminate residual disease in a 4T1 breast tumor model of residual disease. (A) Bioluminescent imaging of luciferase-expressing 4T1 breast tumors treated with no T cells, intravenously administered T cells, a T cell bolus injected into the tumor resection cavity (both unstimulated and prestimulated before injection), and biomaterial scaffold−delivered T cells. (B) Kaplan−Meier survival curves for these groups, showing 100% survival for mice treated with the biomaterial scaffold−deployed T cells. All other groups showed tumor recurrence at varying time points. *Reproduced with permission from Stephan SB, Taber AM, Jileaeva I., Pegues EP, Sentman CL, Stephan MT. Biopolymer implants enhance the efficacy of adoptive T-cell therapy. Nat Biotechnol. January 2015;33(1):97−101, Nature Publishing Group.*

cavity using this macroporous scaffold system loaded with stimulatory microparticles, resulting in no recurrences. In contrast, intravenous injection of T cells had a metastatic relapse rate equal to negative controls, whereas injecting prestimulated T cells directly into the resection cavity had only a modest effect (improving median survival versus controls by an additional 11 days, but not preventing recurrence) (Fig. 41.13) [86]. T cells delivered to the resection cavity via the biomaterial scaffold were found to proliferate at the resection site (167-fold versus injected, prestimulated cells delivered without the scaffold) and maintain a nonexhausted phenotype, whereas intravenously injected T cells accumulated in the spleen and liver instead. In addition, the use of the polymeric scaffold to deliver NKG2D CAR-transduced T cells in a mouse model of stage 3 ovarian carcinoma with peritoneal metastases produced complete tumor clearance in 6 of 10 mice whereas locally injected T cells were unable to show tumor eradication in any animals. The efficacy of this system in preclinical models of improperly resected or metastatic cancer illustrates the potential for a biomaterials approach to provide localized delivery of antitumor immune cells while enabling their proliferation and activation in the face of an adverse tumor microenvironment.

CONCLUSION

The unprecedented scale and scope of ongoing investments in immunotherapy by pharmaceutical companies illustrate the excitement regarding this area [87]. Despite this heavy investment, thus far single-modality immunotherapies such as ICIs have proven effective in only a small fraction of cancer patients. Although rational combinations of immunotherapies that affect multiple points in the cancer-immunity cycle have been shown to be more efficacious, our understanding of cancer immunobiology is still far from complete. Nonetheless, rapid progress witnessed in the field of cancer immunotherapy justifies confidence that increasingly effective immunotherapies can be designed as the components required for a robust antitumor response become increasingly clear. The ability of engineered biomaterial systems to control the spatiotemporal distribution of cells and bioactive factors will allow them to have an important key role in learning how biomaterial−immune system interactions work, but then also to exploit these characteristics to shape an evolving anticancer immune response. It remains to be seen whether the most successful biomaterials-based cancer immunotherapies will be used to improve current therapies such as targeting immune-modulating antibodies and/or adoptively transferred immune cells more effectively to the tumor, enhancing the generation of antigen-specific immune effector cells through better priming, or curtailing the adverse tumor microenvironment. Perhaps an as yet−undiscovered pathway may

present another opportunity for engineered systems to address. Regardless, the continued development of safer and more effective immunotherapies will depend on the effective integration of ideas across a broad spectrum of disciplines and collaboration between scientists and clinicians to allow for the effective translation of these technologies to the clinic.

List of Acronyms and Abbreviations

APCs antigen presenting cells
CAR T-cell Chimeric Antigen Receptor T-cell
CCL20 Chemokine (C-C motif) ligand 20
CpG cytosine-guanosine oligodeoxynucleotides
CTLA-4 Cytotoxic T lymphocyte-associated antigen-4
DCs Dendritic cells
DOPE 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOTMA 1,2-di-O-octadecenyl-3-trimethylammonium propane
EPR effect Enhanced permeation and retention effect
FDA Food and Drug Administration
GM-CSF Granulocyte colony stimulating factor
HER2 Human estrogen receptor 2
ICIs Immune Checkpoint Inhibitors
ICMVs Interbilayer-crosslinked multilamellar vesicles
IFN-gamma Interferon gamma
IL Interleukin
IL-12 Interleukin 12
IL-1α Interleukin 1α
IL-2 Interleukin 2
LPS Lipopolysaccharide
MDSCs Myeloid-derived suppressor cells
MPL-A Monophosphoryl lipid A
MPS Mononuclear phagocytic system
MSRs mesoporous silica rods
NK cells Natural killer cells
NLGs Nanolipogels
OVA ovalbumin
PD-1 Programmed cell death protein-1
PDS Pyridyl disulfide
PLG Poly(lactide-*co*-glycolide)
Poly I:C Polyinosinic−polycytidylic acid
PRR pattern recognition receptor
RGD Arginylglyclaspartic acid
RNA-LPX RNA liposome complexes (lipoplex)
siRNA Short interfering RNAs
STAT3 Signal transducer and activator transcription 3
TAMs Tumor-associated macrophages
tdLNs Tumor-draining lymph nodes
Th1/Th2 T-helper 1/T-helper 2 adaptive immune response
TLR Toll-like receptor
Tregs T-regulatory Cells
TRP2 Tyrosinase-related protein-2

Glossary

Active nanoparticle targeting Nanoparticle targeting applications that require the surface conjugation of a targeting moiety (i.e., ligands, antibody) to target nanoparticles to a desired cell or location.

Adaptive immune system The response of antigen-specific lymphocytes to antigen, including the development of immunological memory. Adaptive immune responses are generated by the clonal selection of lymphocytes. Adaptive immune responses are distinct from innate and nonadaptive phases of immunity, which are not mediated by clonal selection of antigen-specific lymphocytes.

Adjuvant An adjuvant is any substance that enhances the immune response to an antigen with which it is mixed.

Adoptive T-cell therapy A specific immunotherapy strategy which involves the isolation and ex vivo expansion of tumor-specific T cells, and then reinfusion to the patient.

Antigen presenting cells Highly specialized cells that can process antigens and display their peptide fragments on the cell surface together with molecules required for T-cell activation. The main antigen-presenting cells for T cells are dendritic cells, macrophages, and B cells.

Autoimmune responses An adaptive immune response directed at self-antigens is called an autoimmune response; likewise, adaptive immunity specific for self-antigens is called autoimmunity

B Cell One of the two major types of lymphocyte. The antigen receptor on B lymphocytes, usually called the B-cell receptor, is a cell-surface immunoglobulin. After activation by antigen, B cells differentiate into cells producing antibody molecules of the same antigen specificity as this receptor.

Cancer vaccine A specific immunotherapy strategy that aims to train the immune function to recognize and destroy cancer cells. This is typically done by providing both an antigen expressed by the cancer cells and an adjuvant to stimulate generation of T cells specific to that cancer antigen.

Chemokines Chemokines are small chemoattractant proteins that stimulate the migration and activation of cells, especially phagocytic cells and lymphocytes. They have a central role in inflammatory responses.

Clonal T cell expansion Clonal expansion is the proliferation of antigen-specific lymphocytes in response to antigenic stimulation; it precedes their differentiation into effector cells. It is an essential step in adaptive immunity, allowing rare antigen-specific cells to increase in number so that they can effectively combat the pathogen that elicited the response.

Corona A serum protein shell that forms around a nanoparticle, especially charged nanoparticles, after introduction into a protein-rich environment such as blood.

Costimulatory molecules The proliferation of lymphocytes requires both antigen binding and the receipt of a costimulatory signal. Costimulatory signals are delivered to T cells by the costimulatory molecules B7.1 and B7.2, related molecules that are expressed on the surface of antigen presenting cells, and which bind the T cell surface molecule CD28.

Cytokines Cytokines are proteins made by cells that affect the behavior of other cells. Cytokines made by lymphocytes are often called lymphokines or interleukins, but the generic term "cytokine" is used in this book and most of the literature. Cytokines act on specific cytokine receptors on the cells that they affect.

Cytotoxic T cells or lymphocyte T cells that can kill other cells are called cytotoxic T cells. Most cytotoxic T cells are major histocompatibility complex class I–restricted CD8 T cells, but CD4 T cells can also kill in some cases. Cytotoxic T cells are important in host defense against cytosolic pathogens.

Dendritic cells Dendritic cells, also known as interdigitating reticular cells, are found in T-cell areas of lymphoid tissues. They have a branched or dendritic morphology and are the most potent stimulators of T-cell responses. Nonlymphoid tissues also contain dendritic cells, but these are not able to stimulate T-cell responses until they are activated and migrate to lymphoid tissues. The dendritic cell derives from bone marrow precursors. It is distinct from the follicular dendritic cell that presents antigen to B cells.

Effector immune cells Armed effector cells, most often effector T cell, that can be triggered to perform their effector functions immediately upon contact with cells bearing the peptide–major histocompatibility complex for which they are specific. They contrast with memory T cells, which need to be activated by antigen-presenting cells to differentiate into effector T cells before they can mediate effector responses.

Enhanced permeation and retention effect A phenomenon by which molecules of a certain size, typically nanoparticles and/or drugs, tend to accumulate in tumor tissue more so than they do in normal tissue. Many believe that this is a result of leaky tumor vasculature and inadequate lymphatic drainage of solid tumors.

Helper T cell A class of T cells, a specific subclass of CD4+ T cells, that can help B cells make antibody in response to antigenic challenge. The most efficient helper T cells are also known as Th2 cells, which make the cytokines interleukins-4 and 5.

Hydrophilicity/hydrophobicity The physical property of a material to either repel or promote water from binding to its surface.

Immunoediting A dynamic process by which tumors survive attack by the immune system. Typically described as three phases: elimination of immunologically susceptible cells, equilibrium, and finally, immunologic escape.

Immunogenic Any molecule that can elicit an adaptive immune response on injection into a person or animal is called an immunogen, and thus is classified as being immunogenic. In practice, only proteins are fully immunogenic because only proteins can be recognized by T lymphocytes.

Immunologic escape The point at which a tumor is no longer susceptible to immune surveillance and begins to progress in terms of growth and malignancy.

Immunologic memory When an antigen is encountered more than once, the adaptive immune response to each subsequent encounter is speedier and more effective, a crucial feature of protective immunity known as immunological memory. Immunological memory is specific for a particular antigen and is long-lived.

Immunosuppressive A characteristic of something that promotes the inhibition or downregulation of immune responses.

Immunotherapy The prevention or treatment of disease by using or stimulating components of the immune system.

Innate immune system Cells that are responsible for the early phases of the host response to an injury or immunologic insult in which a variety of innate resistance mechanisms recognize and respond to the presence of a pathogen. Innate immunity is present in all individuals at all times, does not increase with repeated exposure to a given pathogen, and discriminates between a group of related pathogens.

Interferons Cytokines that can induce cells to resist viral replication. Interferon-alpha and interferon-beta are produced by leukocytes and fibroblasts, respectively, as well as by other cells, whereas interferon-gamma is a product of CD4 Th1 cells, CD8 T cells, and natural killer cells. IFN-gamma has the activation of macrophages as its primary action.

Interleukins A generic term for cytokines produced by leukocytes. The more general term "cytokine" is often used, but the term "interleukin" is used to name specific cytokines such as interleukin-2.

Macrophages Macrophages are large mononuclear phagocytic cells important in innate immunity, in early nonadaptive phases of host defense, as antigen-presenting cells, and as effector cells in humoral and cell-mediated immunity. They are migratory cells deriving from bone marrow precursors and are found in most tissues of the body. They have a crucial role in host defense.

Monoclonal antibodies Antibodies made from the same clonal cell line and have monovalent affinity, meaning that they bind the same epitope (the part of the antigen that is recognized by the antibody).

Mononuclear phagocytic system A network of phagocytic cells located in the reticular connective tissue that are highly responsible for the clearance of nanosized and microsized materials from the blood.

Myeloid-derived suppressor cells A heterogeneous group of immature myeloid immune cells that have been shown to induce significant immunosuppressive effects, especially in pathogenic situations such as in chronic infections or cancer.

Nanomedicine The applications of nanotechnology in medicine.

Nanotechnology The manipulation and engineering of materials that are in the nanometer-size range, typically less than 100 nm.

Natural killer cells Large granular, non-T, non-B lymphocytes that kill certain tumor cells. Natural killer cells are important in innate immunity to viruses and other intracellular pathogens, as well as in antibody-dependent cell-mediated cytotoxicity.

Passive nanoparticle targeting Nanoparticle targeting applications that use the nature physiochemical properties (i.e., size, shape, and surface charge) to target nanoparticles to a desired cell or location.

Pattern recognition receptors Receptors that bind to pathogen-associated molecular patterns, which are typical of bacteria or many commercially used adjuvants.

PEGylation The process of attachment of poly(ethylene glycol) to molecules, nanoparticles, or macrostructures.

Phagocytosis/endocytosis The internalization of particulate material by cells. If the material is being taken up by a phagocytic cell via invagination of the cellular membrane to form a phagosome, it is referred to as phagocytosis. If the material is taken up by a nonphagocytic cell through membrane invagination to form a vacuole, it is referred to as endocytosis.

Plasmacytoid dendritic cells A class of dendritic cells that circulate in the blood and are found in peripheral lymphoid organs (i.e., spleen, lymph node).

RNA-lipoplex nanoparticles A complex of RNA and lipid material to form liposome-like nanoparticle.

Self-antigens By convention, natural antigens in the body of an individual are called self-antigens. Lymphocytes are screened during their immature stages for reactivity with self-antigens; those that respond undergo apoptosis.

Spatiotemporal delivery Delivery of drug to its optimal location and with optimal time kinetics.

Subunit vaccine A vaccination strategy that uses only part of the disease-causing pathogen. Cancer subunit vaccines typically involve the stimulation of an immune response against a single antigen known to be associated with the cancer cells.

T-helper 1 (Th1)/T-helper 2 (Th2) adaptive immune response Th1 immune responses are adaptive immune responses that are primarily driven by Th1 cells. Th1 cells are a subset of CD4 T cells that are characterized by the cytokines they produce. They are mainly involved in activating macrophages and are sometimes called inflammatory CD4 T cells. Th2 immune responses are adaptive immune responses typically driven by Th2 cells. Th2 cells are a subset of CD4 T cells that are characterized by the cytokines they produce. They are mainly involved in stimulating B cells to produce antibody and are often called helper CD4 T cells.

Therapeutic cancer vaccine Stimulation of the immune system so as to allow for the targeted attack of antigens present on cancer cells, typically through by generating tumor antigen−specific T cells.

Toll-like receptor (TLR) agonists Any agonist aiming to target a TLR. TLRs are a common pattern recognition receptor on antigen presenting cells and other innate immune cells. Binding and activation of TLRs promote inflammatory effects and stimulation of the cell. There are numerous TLRs, each with a different activation potential. They are all named TLR, followed by a number, as in TLR-4.

T-regulatory cells Regulatory or suppressor class of T cells that can inhibit T-cell response.

Tumor interstitium The space within a tumor that is not taken up by cancer cells; essentially all of the remaining space within a tumor.

Tumor microenvironment An intricate network of cancer cells, tumor stroma, and immune cells within a solid tumor that generally promote an overall immunosuppressive state.

Tumor stroma The fibroblast, vasculature endothelial cells, pericytes, and other structural proteins that make up the extracellular matrix component of a tumor.

Tumor-associated macrophages Macrophages found in close proximity or within solid tumors. Tumor-associated macrophages have been shown to induce significant protumor and antitumor effects and induce major immunosuppressive effects.

Tumor-specific antigens An antigen that is overexpressed or specifically located on or within tumors. Often this is an antigen that is unique to the tumor, thus providing a targetable feature.

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CHAPTER

42

Gene Editing in Regenerative Medicine

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GENOME EDITING TOOLS

Genome editing can be defined as the modification of the genome within a living cell through the insertion, deletion, or replacement of one or more segments of DNA. Currently, the commonly accepted view of genome editing entails genome modifications via site-specific nucleases. Although the current view is valued, it is also important to include other genome editing approaches that do not use targeted nucleases, because many downstream applications in regenerative medicine require much higher efficiency editing rates than site-specific editing can supply. These approaches include gene insertion, recombination, and translocation.

Targetable Nucleases

All genome editing approaches that use targeted nucleases share a similar mechanism. The function of targeted nucleases is to induce a double-strand break within the cellular genome at a particular site. After the cleavage is established, inherent cellular DNA repair mechanisms are initiated to fix the double-strand break spontaneously. Three major types of targetable nucleases have been developed and widely applied within the genomic engineering field: clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated systems (CRISPR-Cas), transcription activator-like effector nucleases (TALENs), and zinc-finger nucleases (ZFNs). Among these three systems, CRISPR has attracted considerable attention owing in part to its simplicity, although great work has also been performed using TALENs and ZFNs. Because the three systems share the same DNA repair mechanisms inherent in the cell, therapeutic concepts investigated using one system could be pursued via the other two if certain conditions are met.

Clustered Regularly Interspaced Short Palindromic Repeats

Genes that can be targeted by a CRISPR system are CRISPR-Cas genes. This system, which was first noticed (but not immediately pursued) in *Escherichia coli* by Ishino et al. in 1987 [1], involves repeated sequences of DNA that do not appear in tandem. As time went on and more repeat sequences were identified and characterized in different strains of archaea, bacteria, and mitochondria [2], the repeat sequences became a focus of interest, with the term "CRISPR" being coined in 2002 by Jansen and Mojica and the involvement of Cas proteins being observed at the same time [3].

CRISPR-Cas systems are thought to be parts of a primordial immune system in which bacteria acquire immunity to certain viruses by inserting a fragment of the viral genome into a CRISPR locus. The bacterial immune process basically consists of chopping up the genome of a viral invader into small fragments of approximately 20 bases. Focusing on a single fragment, it will be placed into a special location in the bacterial genome. The locus, now modified with the viral gene fragment, will be transcribed and processed into CRISPR-RNA (crRNA), which we will refer to as guide RNA (gRNA) when discussing applied genome editing.

*Author was at Rutgers University when the chapter was written.

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Cas proteins are restriction enzymes (endonucleases) that have a tunable recognition sequence. The enzyme specificity is determined by the crRNA (gRNA) introduced earlier. The Cas protein binds to a region on the 3′ side of the crRNA (gRNA) known as the "scaffold sequence," leaving a section at the 5′ end of the gRNA, termed the "spacer sequence," free to act as a probe for locating homologous regions within DNA (Fig. 42.1). In wild-type bacterial systems, the crRNA is used to identify DNA as foreign; in engineered systems, the gRNA is used to locate a region in the genome for editing in a site-specific way. After binding of the Cas-bound crRNA or gRNA spacer sequence to a homologous region of DNA, the Cas protein will cleave the DNA in one of several ways. Three different paradigms for gene editing with CRISPR will be discussed subsequently: double-stranded breaks, nickases, and homology-directed repair.

Knockouts via Double-Strand Breaks

Isolated cells can be made to express specific gRNA sequences that can be used to target other DNA sequences for knockout. There are three requirements for the transcribed gRNA: (1) It must have a scaffold sequence that will bind to a given Cas. Cas is inactive unless gRNA is bound. (2) The target sequence must be unique; otherwise, several genes may be bound and produce off-target effects. (3) The target must be just upstream of a protospacer-adjacent motif, also known as a PAM sequence. (For discussion purposes, we will focus on a specific Cas: Cas 9 from *Streptococcus pyogenes*, also known as spCas9. The CRISPR/spCas9 system is one of the most well-characterized and widely used gene editing systems.) The PAM sequence for spCas9 is 5′-NGG-3′. The PAM sequence is an absolute requirement for this system: If the spCas9/gRNA binds to a target sequence that is not next to a PAM, there will be no cleavage by spCas9. However, with the PAM present, cleavage will take place three to four bases upstream of the PAM (Fig. 42.2). SpCas9/gRNA can bind to any portion of the DNA with a PAM, but it is the matching with the spacer sequence that determines whether DNA cleavage will take place.

The spCas9 protein has six domains, including two endonuclease domains: HNH and RuvC. If there is a good match between the gRNA spacer sequence and the target DNA sequence, a conformational change in the Cas9 occurs, thereby activating HNH (which cleaves the target DNA strand) and RuvC (which cleaves the nontarget strand) domains. A double-strand break is thereby introduced.

The double-strand break will be repaired by the cell in one of two ways: nonhomologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is the more common of the two pathways in the cell. It is also more error-prone because insertions and deletions can easily occur, and these can cause frameshift mutations. The result of a frameshift can cause codons to be translated differently, as in different amino acids being assembled onto a growing

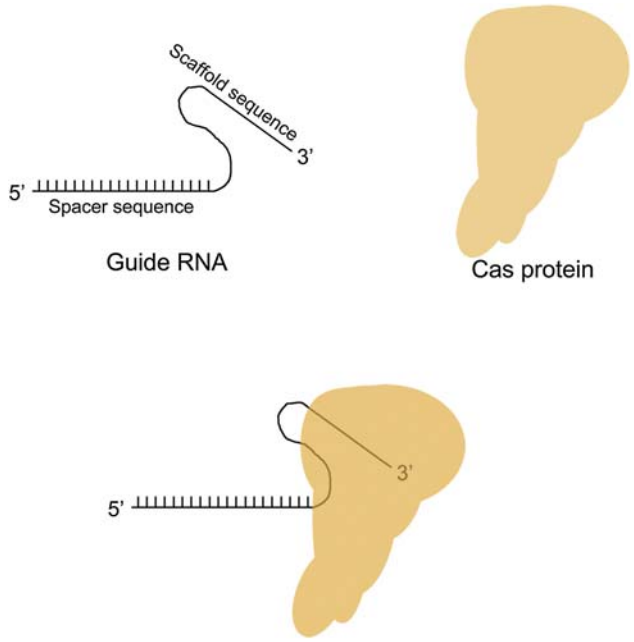

FIGURE 42.1 The DNA recognition and cleavage functions carried out in the clustered regularly interspaced short palindromic repeats (CRISPR) gene editing systems come from a complex consisting of a CRISPR-associated systems (Cas) protein interacting with a CRISPR RNA (crRNA). The crRNA, known as guide RNA in applied gene editing, consists of a scaffold sequence that will bind to the Cas protein, and a spacer sequence that will extend from the complex, allowing it to interact with chromosomal DNA as a probe. The wild-type Cas protein has domains that are responsible for inducing double-stranded breaks in DNA after homology with the spacer sequence has been established.

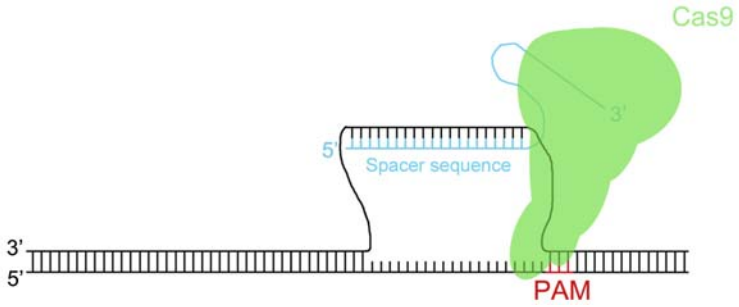

FIGURE 42.2 Interaction of CRISPR-associated systems 9− guide RNA (Cas9/gRNA) with double-stranded DNA. Although a homologous DNA sequence may be recognized by a Cas9/gRNA complex, DNA cleavage will only take place if a protospacer-adjacent motif (PAM) sequence is present in the DNA. It is the PAM sequences, which appear in numerous, nearly evenly spaced copies in the DNA, that are referred to by the CRISPR acronym (**C**lustered **R**egularly **I**nterspaced **S**hort **P**alindromic **R**epeats).

polypeptide, the elimination of a stop codon, or the introduction of a premature stop. In each of these cases, a nonfunctional protein is likely, so this type of DNA repair often results in knocking out a gene by lowering or halting the production of a functional protein product.

HDR has a much higher fidelity, but it is not used as much in the cell. We will return to HDR in a moment.

Nickases

One problem with generating knockouts via double-strand breaks is that despite using a 20-base recognition (spacer) segment on the gRNA, sequences with near-homology may also be cut. One logical solution to this problem would be to use gRNAs with longer spacer sequences. The problem with this idea is that the CRISPR-Cas system is set up for approximately 20-nucleotide spacers, so using a gRNA that has a 40-nucleotide spacer sequence would not work well. An alternative approach is to modify the Cas protein so that it cuts only one DNA strand. By creating a Cas9 with an inactivated HNH domain, or a Cas9 with an inactivated RuvC domain, and by loading the mutated Cas9 protein with respective gRNAs that are specific to separate areas on one of the two strands of DNA, one can essentially require homology at 40 nucleotides (20 for each of the two gRNAs) before the formation of a double-strand break (Fig. 42.3). Only one type of mutated Cas9 is required: HNH-mutated or RuvC-mutated. Unmutated HNH cuts the strand that pairs with the guide RNA, while unmutated RuvC cuts the strand with the PAM sequence. It is through the selection of guide sequences with spacers complimentary to sequences on each of the host DNA strands that is used to generate what amounts to a staggered double-strand break.

The single-stranded breaks introduced by the mutated Cas proteins are known as "nicks"; hence the term "nickases" used to describe the proteins. Nicks are usually repaired quickly by the HDR system and with high fidelity. With the Cas nickase system, a double-nick, in which both strands are cut, will only occur if both of the spacer sequences line up with good homology.

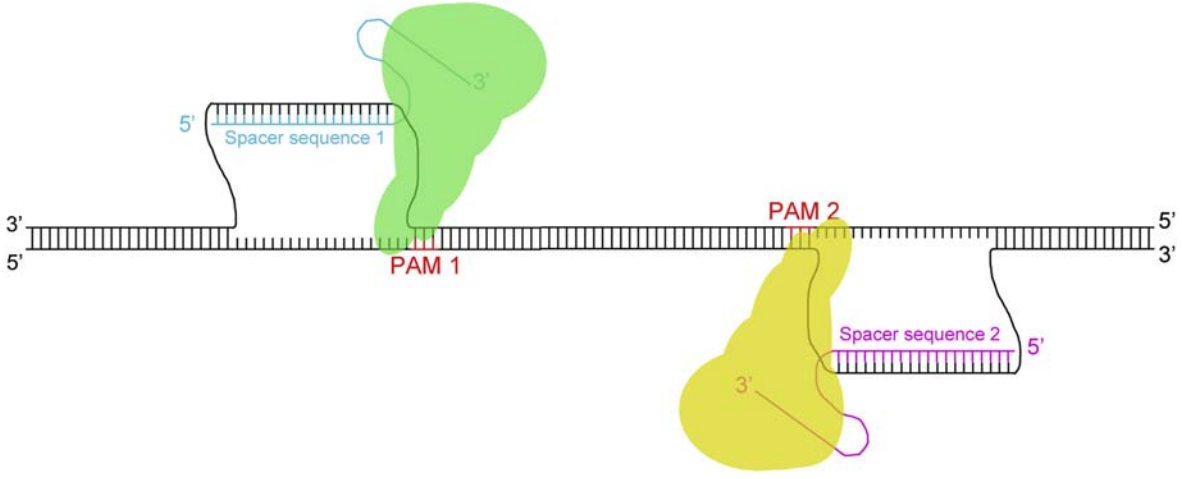

FIGURE 42.3 Nickases working simultaneously to generate single-stranded breaks surrounding a targeted region of DNA. The requirement of homology matching in two distinct guide RNA spacer sequences improves specificity over wild-type clustered regularly interspaced short palindromic repeats (CRISPR)-associated systems (Cas), resulting in a reduction in off-target cleavage. *PAM*, protospacer-adjacent motif.

Homology-Directed Repair

HDR is how CRISPR is used for specific gene edits, such as altering the identity of a single base. Three components are requisite to this system: a Cas protein such as spCas9 or Cas9 nickases, gRNA, and a repair template with homology arms.

The mechanism used for this precise form of gene editing is similar to what has already been covered, except that DNA (linear or plasmid) containing the desired insertion or repair is codelivered with the Cas9/gRNA. Consider a linear repair template designed to correct a point mutation in the genome. Although only one base pair (bp) will differ from the genomic sequence, the length of the repair template must be longer than one bp. The bases to the left and right of the repair bp, called the "homology arms," will have the same sequence as the genomic DNA. The length of each depends on the size of the insert (Fig. 42.4).

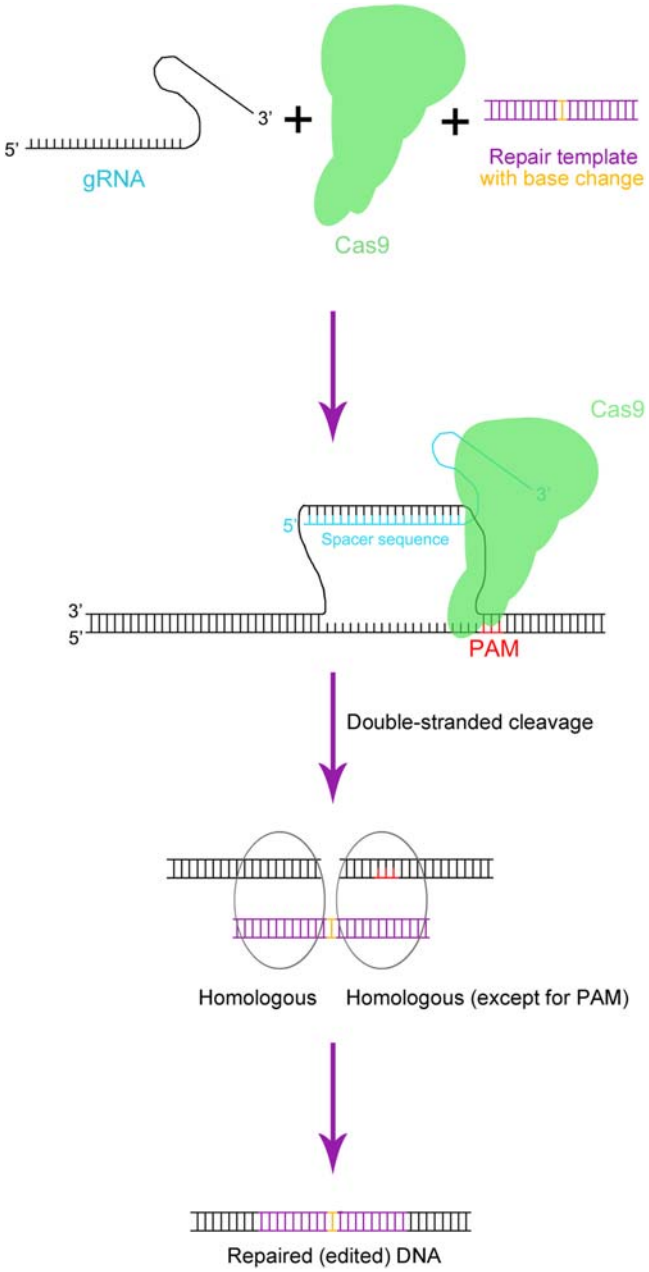

FIGURE 42.4 Homology-directed repair. In this scheme of genome editing, clustered regularly interspaced short palindromic repeats (CRISPR)-associated systems (Cas)—guide RNA complexes (Cas9 shown here) are codelivered with a repair template. The template will be introduced into the genome via homologous recombination after the introduction of a double-stranded break. *PAM*, protospacer-adjacent motif.

It is important for the repair template not to contain a PAM sequence; otherwise it would be cut by the Cas proteins. If the section to be inserted happens to contain a PAM sequence, the insertion sequence should be reengineered with a silent mutation to remove the PAM. For example, an AGG sequence, which codes for arginine and is also is a Cas9 PAM sequence, could be modified to AGA, which still codes for an arginine but is no longer a PAM sequence.

Recall that the HDR, while having high fidelity, is not used by the cell as much as NHEJ, which means NHEJ will still be at work. Because somatic mammalian cells have two alleles, the result of this type of gene editing for a given cell will be a combination of:

- wild-type,
- NHEJ-mutated, and/or
- HDR-inserted (repaired) alleles.

It is therefore imperative for the researcher to perform a screen to isolate HDR−HDR clones.

SpCas 9 Variants and Orthologues

Although the SpCas9 system has been widely accepted for laboratory applications worldwide, some limitations still must be overcome. For example, SpCas9 depends on the specific (*S. pyogenes*) PAM sequence 5′−NGG−3′, and the cleavage site must be close to this PAM. Although NGG is abundant throughout the mammalian genome, the appearance of the sequence might not be close enough to the desired target to effect the desired modification. To address this issue, multiple novel Cas9 variants that use different PAM sequences have been engineered or discovered. Benjamin et al. developed a series of Cas9 variants by mutating the particular site of SpCas9 (D1135/R1335/T1337 or D1135/G1218/R1335/T1337) or screening various Cas9 orthologues from nature [4,5]. The PAM library has been expanded to include 5′-NGCG-3′ (SpCas9 VRER variant), 5′-NGAG-3′ (SpCas9 EQR variant), 5′NGAN-3′ (SpCas9 VQR variant), 5′-NNAGAAW-3′ (Cas9 from *Streptococcus thermophilus* [St1Cas9]), and 5′-NNGRRT-3′ (Cas9 from *Staphylococcus aureus* [SaCas9]) [4]. These Cas9 variants and orthologues provide great convenience to scientists by expanding the editable areas within the genome.

Off-target effects pose a potential risk to future therapeutic applications of CRISPR [6,7]. These occur when the nuclease induces a double-strand break at an unwanted site, leading to unwanted cellular changes such as transformation. To reduce the possibility of off-target effects, SpCas9 has been mutated by rational design with apparent success. One such set of mutations, designed to reduce potential interactions between SpCas9 and the phosphate backbone of DNA through direct hydrogen bonds, produced what is referred to as the *S. pyogenes* Cas9 High-Fidelity variant 1 (SpCas9-HF1). (The specific mutations to the wild-type SpCas9 were N497 A, R661 A, Q695 A, and Q926 A [8].) SpCas9-HF has editing activity that is comparable to wild-type SpCas9, but off-target events have been nearly eliminated. Another set of mutations designed to reduce the affinity between a positively charged groove (located between the HNH, RuvC, and PAM-interacting domains) and the negative charges of genomic DNA have served to neutralize positively charged residues in the Cas9 (K810 A, K848 A, K1003 A, and R1060 A) [9]. The mutated enzyme was named “enhanced specificity” SpCas9 (eSpCas9), and like SpCas9-HF1, it has been shown to have activity similar to the wild-type enzyme but with a clear reduction in off-target events.

A novel CRISPR system from the genera *Prevotella* and *Francisella*, called Cpf1, has introduced several specific features to these systems [10]. Usually, the double-strand breaks introduced by a Cas protein are blunt ended. The Cpf1 system is different in that it creates overhangs of four to five bases [10]. The overhangs present an opportunity to increase the efficiency of gene insertion. In addition, the Cpf1-associated system does not require the transactivating crRNA to perform the DNA interference function [10]. Cpf1 is able to process pre-crRNA into crRNA without relying on host RNase, which makes it possible to edit multiple genes simultaneously. Because of the large size of a single gRNA expression cassette (∼400−500 bp), for SpCas9, each single gRNA must be placed in different vectors or driven by a different set of promoters [11,12]. However, thanks to the feature of Cpf1 being guided by a small (39-nucleotide) crRNA, a single promoter within one vector is enough to drive many crRNA together. Published experiments have achieved simultaneous modification of four different targets by using Cpf1 with a single construct [13] (Fig. 42.5).

Another interesting system employs CRISPR-C2c2, which comes from the bacterium *Leptotrichia shahii* [14]. Instead of working on DNA, CRISPR-C2c2 can serve as an RNA-guided RNase that can cleave certain single-stranded RNAs via pairing with a 28-nucleotide sequence in the crRNA. This system could be used to downregulate gene expression via posttranscriptional knockdown, similar to RNA-induced silencing complex−mediated RNA

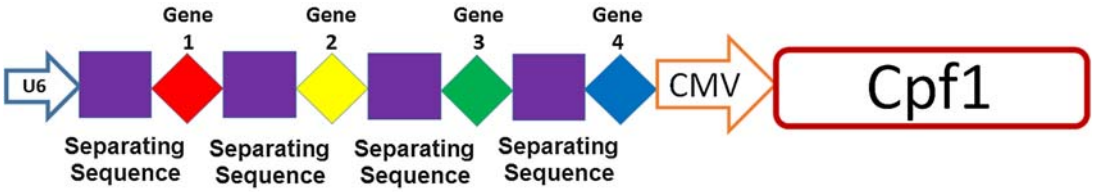

FIGURE 42.5 Schematic outlining the components of a multiplex gene pre−clustered regularly interspaced short palindromic repeats RNA array. The outline represents a single construct consisting of guide sequences for four genes and Cpf1. Two promoters are included: the U6 promoter directs transcription of the four guide sequences and the cytomegalovirus promoter drives transcription of the Cpf1 nuclease gene. *Figure adopted from Zetsche B, Heidenreich M, Mohanraju P, Fedorova I, Kneppers J, DeGennaro EM, et al. Multiplex gene editing by CRISPR-Cpf1 using a single crRNA array. Nat Biotechnol 2016.*

interference [14]. Moreover, fluorescent labeling of C2c2 makes it possible to tag specific messenger RNA (mRNA) sequences and linking dC2c2 with a splicing factor makes it possible to affect mRNA splicing [15]. However, the function of the system has been shown only in bacteria. Further investigation is necessary before it can be used reliably in mammalian cells.

Transcription Activator-like Effector Nucleases

TALENs are chimeric proteins which contain two functional domains: a DNA-recognition transcription activator-like effector (TALE) and a DNA nuclease domain. They work for gene editing by recognizing a specific sequence, which the user can design, and introducing a double-stranded break with an overhang. TALENs therefore serve as a form of customizable restriction enzyme.

The DNA-recognition TALEs used in TALEN technology are secreted by the genus *Xanthomona*, a type of phytopathogenic bacteria [16]. A TALE consists multiple domains, including a translocation domain at the N terminus, a nuclear localization signal and a transcription-activation domain that are close to C-terminus, and multiple repeats in the center that serve to recognize DNA molecules [16]. The repeat sequences are composed of a highly conserved 33− to 34−amino acid sequence, with positions 12 and 13 being variable. The variable portion of the sequence is termed the "repeat variable di-residue" (RVD). By changing the RVD sequence of a specific repeat, that particular repeat can be made to bind a specific nucleotide [17]. As examples, a monomer with an RVD consisting of asparagine-asparagine will bind with a purine, asparagine-isoleucine will bind with adenine, asparagine-glycine will bind with thymine, and histidine-aspartate will bind with cytosine (Table 42.1) [17]. By manipulating the RVDs of its repeated sequences, a TALE can be made to serve as a probe for a specific site within the genome.

The nuclease portion of a TALEN is the catalytically active domain of the restriction enzyme FokI. (The DNA recognition domain has been removed.) FokI can be used in mammalian cells to cut genomic DNA, but it must be dimerized to be functional. Both homodimeric and heterodimeric domains can be used [18].

One challenge in using TALENs is that the enzyme construct is hard to build owing to the numerous repeat sequences that must be customized for targeting. This is why much effort has been directed toward improving the construction of TALENs. One method, called Golden Gate assembly, is widely accepted for its relative simplicity [19]. This method employs type IIS restriction endonucleases, which cleave outside their recognition sequences. By selecting appropriate type IIs endonucleases, the recognition sequence can be removed from the fragment of interest, thus allowing for digestion and ligation in the same reaction mixture. Similarly, because the site where

TABLE 42.1 The Identity of the Variable Sequences (Repeat Variable Di-residues [RVDs]) in a Transcription Activator-like Effector Determine the Nucleotide Targets

RVD Sequence	Nucleotide Bound
Asparagine-asparagine	Purines
Asparagine-isoleucine	Adenine
Asparagine-glycine	Thymine
Histidine-aspartate	Cytosine

the DNA cleavage itself occurs is not sequence-specific (is outside the recognition sequence), the compositions of the resulting overhangs are not dictated by the enzyme, so multiple, specific overhangs can be created simultaneously, allowing for the creation of multiple assemblies.

Compared with the CRISPR system, which requires only the design of a single gRNA, the TALEN system requires significantly greater effort devoted to sequence design and cloning. As a result, CRISPR is the more widely accepted method for genome editing. TALENs have certain advantages over CRISPR in specific situations, though. For example, TALENs can theoretically target any site within the genome, as opposed to CRISPR, which requires the presence of a PAM site. Therefore, when no PAM site is available for a given application, one can still turn to a TALEN system. In addition, TALENs are associated with lower off-target effects versus wild-type SpCas9. For perspective, a study of off-target cleavage by CRISPR-Cas9 (using SpCas9) versus TALENs was performed using six different genes. The TALENs produced no off-target cleavage, whereas half of the applications (three of six) using SpCas9 nucleases yielded off-target events [20]. Published comparisons of TALENs with the high-fidelity forms of CRISPR were not readily available at the time this chapter was written.

Zinc Finger Nucleases

ZFNs are another type of DNA recognition/cleavage construct that acts like a restriction enzyme. They combine the Cys2-His2 zinc finger motif as the DNA recognition domain with the FokI DNA cleavage domain [21]. The Cys2-His2 zinc finger motif consists of a single zinc atom with about 30 amino acids in a β sheet−β sheet−α helix conformation [22]. Each zinc finger contacts three DNA bases through amino acids on the α helix of the motif [23]. The selectivity between a zinc finger and target triplets can be modulated by adjusting the amino acid sequence. A library of zinc-finger domains that can target nearly all of the possible triple nucleotide combinations has been developed [24]. By connecting three to six zinc finger motifs via conserved linker sequences, 9−18 nucleotides will be targeted by a monomer [25]. As with TALENs, because ZNFs use FokI as the nuclease, they are also designed for use as dimers, so a total of 18−36 nucleotides will be targeted.

The primary hurdle for using ZFNs is that of enzyme design and construction. The cloning process used to produce the correct amino acid sequence is labor intensive, but the correct primary sequence is also imperative for achieving high targeting specificity. As a result, the number of studies applying this technique has dwindled since TALENs and CRISPR were introduced to the field. However, unlike the TALENs and CRISPR systems, ZFNs originated from mammalian cells, so there has been less concern regarding immunogenicity. Phase I clinical trials that infused ZFN-modified autologous CD4 T cells as a treatment for HIV have been completed, with the treatments being deemed safe (NCT00842634) [26]. This implies that ZFNs may be suitable for other clinical applications.

Other Genome Manipulation Tools

Transposons and Transposase

There are two types of transposons: class I transposons, also known as retrotransposons, which are transcribed and reverse-transcribed as part of the translocation mechanism, and class II transposons, which do not require transcription−reverse-transcription but use transposases to catalyze the translocation mechanism. The transposons used for genomic engineering are class II. Combined with the enzyme transposase, a transposon can be moved efficiently between plasmid and genome in a cut-and-paste fashion. Transposase acts by recognizing a sequence of inverted terminal repeats (ITRs) that appear at each end of the transposon and introducing a pair of double-stranded breaks in the DNA. A gene flanked by ITRs can be cut out of a plasmid and transferred to a site within the genome that is flanked by the same ITRs. This method can also be used to delete genes that appear between two ITRs sites in the genome. There is no size limit for transposons. This system has been used, for example, to reprogram primary cells into induced pluripotent stem cells (iPSC) through virus exclusion [27].

Recombinase

The major function of recombinase is to exchange strands between two DNA segments that have partial sequence homology. The most widely used recombinase systems for genome manipulation are Cre-loxP and Flp-FRT. The Cre and Flp enzymes are site-specific recombinases that recognize 34-bp recognition sites. The loxP recognition site contains two 13-bp palindromic sequences and an 8-bp spacer (5′-ATAACTTCGTATA-atgtatgc-TATACGAAGTTAT-3′, [spacer shown in lowercase]) [28]. Similar to loxP, FRT consists of two 13-bp palindromes surrounding an 8-bp spacer (5′-GAAGTTCCTATTC-tctagaaa-GTATAGGAACTTC-3′) [28]. The Cre and Flp recombinases can be used for gene

insertion, excision, inversion, or translocation combined with their respective recognition sites and are often applied in genome engineering [29], such as for generating conditional mutants. The International Knockout Mouse Consortium has been launched to produce mutant murine embryonic stem cells for every gene in the mouse genome (more than 20,000 genes) by incorporating the loxP and FRT sites [30]. Using Cre and Flp, knockout mice for any gene can be produced from these cells.

Integrase

In the past, gene insertion typically was achieved by plasmid integration with stringent antibiotic selection or via infection with lentiviral vectors. Insertions occurred randomly and the copy number, location, and direction of insertion were difficult to control. Homologous recombination with or without a targetable nuclease addressed the problem of directionality, but the size of the inserted gene was limited and integration efficiency was still lacking, especially in primary cells [31].

A technology named "dual integrase cassette exchange" (DICE) provides an alternative route whereby the copy number, location, and direction gene integration are controlled, and with higher efficiency [32]. DICE adopts the phage integrases phiC31 and Bxb1, which have the ability to insert a gene into their own recognition sites by unidirectional recombination. However, these recognition sites do not exist in the mammalian cell genome. To get around this problem, a "landing pad" was introduced into a safe region of the mammalian genome by integrating those recognition sites through TALEN-assisted homologous recombination. As a result, desired genes could be efficiently implanted into the landing pad by codelivery of the two integrases and donor template. It is claimed that there is no size limitation for the inserted gene [32].

DELIVERY CARGO

The genome-editing tools just described can be delivered into cells via multiple routes based on whether DNA, RNA, or protein is the tool being used.

DNA

DNA is often delivered as a plasmid, which is typically constructed via standard molecular cloning techniques. When properly preserved, DNA has a long shelf life. The efficiency of DNA delivery into cells is the major limiting factor for the associated methods. DNA for genome editing can also be engineered into a viral genome, and plasmids can be adapted for use with viruses for delivery into cells [33]. Although viruses typically generate higher gene delivery efficiencies than do nonviral gene delivery agents, immunogenicity is a concern for in vivo applications, especially if repeated delivery events must be performed.

Another concern regarding the delivery of DNA into cells is that the foreign DNA could integrate into the host genome through homologous recombination. If this occurs in an untargeted fashion, vital host genes may be disrupted or inactivated, tumor suppressor genes may be knocked out, or oncogenes might be activated. Whether targeted or untargeted, genomic integration could cause the encoded nuclease to be expressed in a sustained manner, potentially causing continuous formation of double-strand breaks and serious off-target effects.

RNA

The editing methods that use delivered RNA avoid the possibility of genomic integration. RNA can be produced by cells transfected in vitro, harvested, and applied to cells of interest in subsequent experiments. With RNA delivery, the efficiency of enzyme production may be enhanced because genetic materials do not have to reach the nucleus and the transcription step is eliminated. However, RNA is more difficult to handle and store than is DNA because RNases are abundant in the cell, on the skin, and throughout most research environments. The immunogenicity of RNA produced in vitro should also be considered [34].

Proteins

Instead of delivering genetic materials, delivering proteins into cells for gene editing is now a commercially available option. Multiple CRISPR Cas9 protein delivery kits exist on the market; TALENs and ZFNs can also be

delivered into cells. For the CRISPR system, the ribonucleoprotein complexes are formed in vitro by combining a Cas nuclease with the appropriate gRNA, and the constructs are delivered into cells without fear of genomic integration. Another advantage to delivering proteins instead of (deoxy)ribonucleotides is that the amount of enzyme that will reside within the cell is more precisely controllable.

DELIVERY METHODS

Microinjection, gene gun, electroporation, and hydrodynamic delivery are all methods that have been used to deliver genome editing tools into cells [35–38]. These methods are not widely used because they either need specialized equipment and experienced professionals (microinjection) or they are associated with cell or tissue damage (gene gun, electroporation, and hydrodynamic delivery). These methods are also restricted in their ability to reach host tissues in vivo.

Chemical agents such as cationic lipids, polymers, and dendrimers have been widely used for gene delivery, but they are associated with gene delivery efficiencies that are generally lower than those of viral vectors. However, chemical delivery methods are still preferred compared with viral methods in some cases owing to their ease of preparation, lower immunogenicity, and lower expense [39].

All of the chemical gene delivery methods can be applied to genome editing because there is no chemical difference between plasmids encoding targetable nucleases and plasmids encoding other genes. However, different mechanisms may be involved for the delivery of proteins. Cationic liposomes have been used to this end with relative success. The delivery of Cas9–gRNA complexes or TALENs via cationic lipids is evident by the multiple commercially available agents that are in the marketplace [40,41]. The cationic polymer poly(ethylenimine) has been used to coat self-assembled DNA cages to delivery Cas9/gRNA complexes successfully into mammalian cells [42]. Cell-penetrating peptides have been used to deliver targetable nuclease proteins, including Cas9 and TALEN [43,44]. Interestingly, ZFN has inherent cell-penetrating capabilities and can pass into cells without a carrier [45].

Viruses have been used for gene delivery for decades [46]. For gene editing applications, several viruses have been evaluated, including lentivirus [47], adenovirus [48], and adeno-associated virus (AAV) [49]. Lentiviral vectors have been shown to have a high transduction efficiency in multiple primary cell types and are capable of having genes integrated into the genome of the host [39,50]. As mentioned, incorporating a gene encoding a nuclease into the genome can lead to constitutive expression of the enzyme, which can lead to off-target events. Even worse, lentiviral integration occurs randomly, which can potentially induce various malignancies [51,52]. One solution to these problems of integration is to knock out the viral gene that encodes integrase, a technique that has been used to transfer genes encoding ZFNs, TALENs, and Cas9 [20,53]. Another solution is to develop lentivirus-derived particles to carry nuclease proteins. ZFN, TALEN, and Cas9 proteins have been successfully packed into such lentiviral particles [47,54]. Donor templates have been copacked into the particles to achieve targeted DNA insertion and gene correction [54]. It has been reported that this approach has lower off-target activity than traditional delivery methods [47,54].

Adenoviral vectors allow transient transgene expression in both dividing and nondividing cells. These vectors carry a much lower risk of genomic integration than their retroviral counterparts. A set of third-generation adenoviral vectors named "helper-dependent adenoviral vectors" have increased cargo sizes (36 kb, as opposed to ~8 kbp) [55], which provides more versatility for the delivery of donor DNA templates for gene insertion and correction. However, immunogenicity is still a concern, as it is for all virus methods [56], especially when multiple gene delivery events must be carried out.

Like adenovirus, AAV can produce transient transgene expression in both dividing and nondividing cells. In gene delivery, certain AAV serotypes are preferred because their delivered genes will be integrated into the host genome in a targeted fashion. Keeping in mind the previous discussion about problems with constitutively expressed nucleases, recombinant AAV have been produced that lack the viral *rep* gene to lower or eliminate the frequency of genomic integration [57]. Some strains of AAV carry lower immunogenicity concerns for a single administration, but repeated administrations require different serotypes to avoid a secondary immune response. These viruses are relatively small so they have limited DNA loading capacity (~5 kb) [58]. The ZFNs with double monomers [59] are usually compatible with this limitation, but it is difficult to package double TALEN monomers (~3 kb each) or SpCas9 (~4.2 kb) and associated gRNA within a single AAV vector [60]. For in vitro genome editing, this limitation can be overcome by packaging the functional units separately and cotransducing cells. Edited cells can then be screened and selected. However, this is not practical for in vivo editing because the selection step

cannot be performed, and every abnormal cell is expected to be treated. One could reduce the size of the gene encoding the enzyme: a Cas9 orthologue, named *S. aureus* Cas9 (SaCas9), which is 1 kb smaller than SpCas9, was identified [60]. The smaller gene has been successfully copacked with gRNA in a single AAV construct, with precise genome editing still being attainable.

APPLICATIONS OF GENE EDITING IN REGENERATIVE MEDICINE

Stem cells are characterized by two properties: self-renewal, in which they are able to divide numerous times while remaining undifferentiated, and pluripotency, which means they can differentiate down any of the three germ cell lineages (ectodermal, mesodermal, and endodermal). Because of these characteristics, stem cells are attractive progenitors to a broad range of regenerative medicine applications. Stem cells can be harvested and isolated from various tissues such as bone marrow [61], amniotic fluid [62], umbilical cord blood [63], or brain [64]. In addition, they can be induced from differentiated cells by introducing genes encoding specific transcription factors [65]. In contrast to embryonic stem cells, adult-derived stem cells and iPSCs can be harvested or produced autologously, thus eliminating the need for immunosuppression and providing a means of personalized medicine as new applications are developed. As genome-editing technologies develop, gene-edited stem cells may be produced for the repair and regeneration of various tissues and organs [66]. Various applications of genome editing to the regeneration of specific tissues or organs are presented in this section.

Liver

The liver is a vital organ containing numerous enzymes that are involved in a wide range of bioprocesses such as glucose storage and gluconeogenesis, cholesterol synthesis, bile production, urea production, the synthesis of certain blood proteins, red blood cell turnover, and the detoxification of metabolites and drugs (such as ethanol). Because of the importance of this organ and the numerous diseases that can arise to affect its function, the liver is a prime target for regenerative medicine and gene editing therapy.

α1-Antitrypsin (A1AT) deficiency is an autosomal recessive disorder that is caused by a single point mutation (Glu342Lys). Copies of the mutated protein aggregate in the endoplasmic reticulum of hepatocytes instead of being secreted into the blood and body fluids [67,68]. A1AT deficiency may further develop into cirrhosis of the liver and necessitate the need for liver transplantation [69]. ZFNs and PiggyBac transposons have been used in conjunction to correct the point mutation in the *A1AT* gene in human iPSCs derived from a patient with A1AT deficiency [69]. The cells were then differentiated into hepatocyte-like cells, which displayed normal A1AT function. This pioneering work demonstrated the potential for applying genome-editing and iPSC technologies for an autologous cell-based therapy.

Several disorders are related to deficiencies in one or more of the enzymes of the urea cycle, a biochemical pathway that is involved in removing nitrogen from the body. Hyperammonemia is one such condition that can lead to encephalopathy and, potentially, death. Arginase, which is an enzyme of the urea cycle, has a part in ureagenesis. Defects in the gene encoding arginase can lead to hyperammonemia. Gene editing has been used with patient-derived iPSC to restore arginase activity in arginase-deficient patients [70]. In these experiments, CRISPR-Cas9 was used to add an arginase-1 cDNA expression cassette into the first exon of the hypoxanthine-guanine phosphoribosyltransferase locus in the isolated cells. The cells were then differentiated into hepatocyte-like cells, which displayed both arginase activity and ureagenesis [70].

Another potential cause of urea cycle disruption is ornithine transcarbamylase deficiency, caused by a mutation in the associated gene. Gene editing research toward addressing this malady has included a dual AAV system used to deliver Cas9-gRNA and donor DNA separately into newborn mice with a partial deficiency in ornithine transcarbamylase [71]. Results have been weak but encouraging, with correction of the mutated gene in about 10% of hepatocytes and improved survival in treated mice that were given high-protein diets.

Tyrosinemia, which is characterized by a buildup of tyrosine (and some of its breakdown products, depending on the type), is a severe genetic disorder stemming from an inability to break down the amino acid tyrosine. There are three types of tyrosinemia, each characterized by the specific enzyme that is mutated in the degradation pathway (Fig. 42.6). Tyrosinemia type I is caused by a deficiency in the enzyme fumarylacetoacetate hydrolase (FAH), which catalyzes the conversion of fumarylacetoacetate into fumarate plus acetoacetate [72,73]. A deficiency

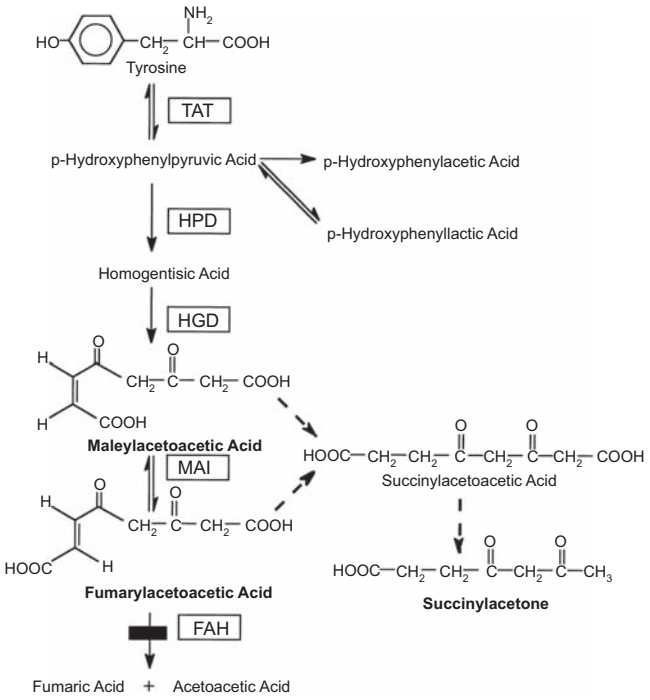

FIGURE 42.6 Partial degradation pathway of tyrosine, highlighting the steps associated with tyrosinemia type I, catalyzed by fumarylacetoacetate hydrolase (FAH). Tyrosinemia type II has a deficiency in the enzyme tyrosine aminotransferase (TAT) and type III tyrosinemia has a deficiency in the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPD). *Figure from Jorquera R, Tanguay RM. Cyclin B-dependent kinase and caspase-1 activation precedes mitochondrial dysfunction in fumarylacetoacetate-induced apoptosis. FASEB J 1999;13(15):2284—98.*

in FAH results in the accumulation of fumarylacetoacetate and maleylacetoacetate, which induce cellular damage [74]. CRISPR-Cas9 has been examined for use as a treatment for hereditary tyrosinemia type I, in which the gene encoding 4-hydroxyphenylpyruvate dioxygenase was deleted. This deletion caused hepatocytes associated with tyrosinemia type I to be converted into hepatocytes that would be associated with the more-benign tyrosinemia type III [75]. Rather than relying on genetic deletion, a different laboratory group used a gene editing approach to deliver mRNA encoding Cas9 via lipofection, along with AAV containing DNA encoding gRNA, to correct mutant *fah* in mice with human hereditary tyrosinemia. This method yielded modest recovery of FAH function in approximately 6% of hepatocytes [76].

Angiogenesis

Angiogenesis is a process in which new capillaries grow from preexisting vessel networks to form more complexed vessel networks [77]. It is essential for the regeneration of tissues and organs if the component cells are to receive oxygen and nutrients and have carbon dioxide and other waste products removed. Therefore, procedures designed to enhance angiogenesis are related to regenerative medicine.

Genome editing using TALENs has been used to induce cells to express hepatocyte growth factor (HGF) to induce angiogenesis [78]. The common theme of editing the genomes of progenitor cells was used on mesenchymal stromal cells isolated from human umbilical cord blood. In this case, a TetOn sequence preceded a gene encoding HGF pUC19-TetOn-*HGF* expression cassette, and the DNA was inserted into chromosome 19. The TetOn sequence was used as a safety measure. It allowed for control of the *hgf* gene by requiring that tetracycline be present for the gene to be transcribed. After the establishment of HGF expression in the cells, the cells were encapsulated in alginate and transplanted to correct a hind limb ischemia model. The entire procedure worked well, yielding angiogenesis in the in vivo model.

Muscle: Muscular Dystrophy

Muscular dystrophy is family of diseases characterized by a gradual loss of muscular strength and control. There are nine forms of the disease, each with its own characteristic set of symptoms and time of onset. Some affect only

TABLE 42.2 Types of Muscular Dystrophy and Some Defining Characteristics [79]

Type	Sexes Affected	Typical Time of First Appearance	Characteristic Muscles Affected
Duchenne	Males	Ages 3–5	Upper legs and pelvis; heart and diaphragm in advanced cases
Becker	Males	Ages 11–25	Upper legs, pelvis; similar to, but milder than, Duchenne
Limb-girdle	Both	Teens to early adulthood	Begins in hips, spreads to shoulders, legs, and neck
Myotonic	Both	Ages 20–30	General: prolonged stiffening of muscles after use
Facioscapulohumeral	Both	Teens to forties	Face, shoulders, and back
Congenital	Both	Present at birth, symptoms by age 2	General weakness, progressing to muscle shortening
Oculopharyngeal	Both	Ages 40–60	Muscles of the eye, face, and throat
Distal	Both	Ages 40–60	Hands, forearms, feet, and lower legs
Emery–Dreifuss	Males	Ages 10 to mid-twenties	Shoulders, upper arms, and lower legs

males whereas others affect either sex. The symptoms of some appear in infancy whereas others present in adolescence or adulthood. The areas affected are also varied and are characteristic of the form of the disease being experienced (e.g., limbs, eyes and throat, face and shoulders, hips, hands, or even heart and diaphragm). A brief description of the types of muscular dystrophy appears in Table 42.2.

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked genetic disease arising from mutations in the *dystrophin* gene, a large gene consisting of 2.4 million bp and containing 79 exons. Exons 45–55 hold the mutational hot spot for the gene. Frameshift mutations lead to dysfunction of the dystrophin protein, which has an important role in skeletal and cardiac muscle function. In the absence of functioning dystrophin, patients experience progressive muscle weakness and wasting. In conventional virus-mediated gene therapy, owing to the size limitations imposed by the viral capsid, the full-length DMD cDNA must be replaced by a truncated complementary DNA (cDNA) that will yield proteins with compromised function, yielding minimal efficacy and a T cell response after gene delivery in vivo. Exon *dys* 51 (from Sarepta Therapeutics, Inc.) was developed using an oligonucleotide exon-skipping strategy [80]; after a controversial argument regarding its efficacy [80], it was approved by the US Food and Drug administration in 2016 as the first drug to treat DMD [81]. One drawback of the treatment, however, is that patients must receive regular intravenous infusions.

Genome editing provides the ability to correct mutations in genes without the need for introducing overly long sequences. Because of this, editing tools hold a different promise than do conventional gene delivery techniques for providing a more complete treatment for cells with mutations to the dystrophin gene. For instance, paired ZFNs have been delivered to DMD patient-derived myoblasts using electroporation, with the purpose of removing exon 51 of the dystrophin gene. This led to restoration of the dystrophin reading frame in approximately 13% of DMD patient mutations [82]. Long et al. [83], Nelson et al. [84], and Tabebordbar et al. [85] used CRISPR-Cas9 systems on the mdx mouse model of DMD to delete the mutated exon 23 from the dystrophin gene, resulting in the restoration of expression of functional dystrophin in the murine skeletal and cardiac muscles, plus enhancements in skeletal muscle function in the mice. Long et al. [83] delivered SpCas9 using AAV9, whereas Nelson et al. [84] and Tabebordbar et al. [85] delivered SaCas9 via AAV8 and AAV9, respectively. Iyombe-Engembe et al. used a pair of gRNAs to target exons surrounding exons 51–53 to bring about their deletion from the *DMD* gene, which restored the correct reading frame with 62% efficiency [86].

The CRISPR-Cas9 system has been delivered by "all-in-one" adenoviral vectors into patient-derived muscle progenitor cells for the purpose of removing mutations from the major *DMD* mutational hot spot [87]. This blanket approach has been asserted to counteract multiple mutations and provide treatment coverage in more than 60% of patients with DMD. Another investigation that targeted the DMD mutational hot spot deleted up to 725 kb using CRISPR-Cas9 with NHEJ. The efforts reportedly restored the function of dystrophin in cardiomyocytes and skeletal muscle cells derived from edited human iPSCs [88].

TALENs have been used to target a point mutation (A > G) associated with muscular dystrophies in iPSCs isolated from a canine golden retriever model of muscular dystrophy. The corrected canine progenitor cells were implanted into immunodeficient mice after ischemia-induced myocardial injury and cardiotoxin-induced hind limb skeletal muscle injury, yielding encouraging levels of regeneration in both cardiac and skeletal muscles [89].

Limb-Girdle Muscular Dystrophy

Limb-girdle muscular dystrophy types 2B and 2D are muscular dystrophies that are induced by mutations in the dysferlin and α-sarcoglycan genes, respectively. The dysferlin nonsense mutation (c.5713C > T; p.R1905X) and the α-sarcoglycan missense mutation (c.229C > T; p.R77C) have been corrected using the CRISPR-Cas9 system in iPSCs isolated from patients with the respective diseases [90].

Another treatment aimed at the dysferlin mutation involved site-specific cDNA insertion via DICE, or TALEN-assisted homologous recombination for insertion precise, carried out in iPSCs isolated from patients with the disease. Expression of dysferlin was restored in the corrected iPSCs [90]. The typical next step would be to reintroduce the cells into the patient with the hope of restoring muscle function. Although the gene corrections were significant, using the corrected cells to restore function is a task that is more easily said than done.

Blood

The β-hemoglobinopathies, including β-thalassemia and sickle cell disease, are a group of genetic blood diseases caused by mutations in the β subunits of hemoglobin (HBB). These mutations are responsible for the decreased production of functional or mature β-globin subunits and therefore reduce the oxygen-carrying capacity of the blood [91]. β-Thalassemia can be caused by different mutations, whereas sickle cell disease is caused by a single DNA base mutation (A > T) in the sixth codon of the *hbb* gene [92,93]. Allogeneic stem cell transplantation is an effective way to treat β-hemoglobinopathies but it is difficult to find a fully matched donor, so immune rejection of the transplanted cells is often a concern. The combination of genome editing with (autologous) stem cell technology has brought about a potentially improved approach to treat these blood disorders.

For instance, a CRISPR-Cas9 system consisting of Cas9 ribonucleoproteins and a homologous, unmutated DNA sequence (carried by AAV) was used to achieve homologous recombination at a site of mutation in the *hbb* gene in isolated hematopoietic stem cells. These *hbb*-corrected cells were further enriched and transplanted into nonobese diabetic, severe combined immunodeficiency mice, yielding edited cells in the bone marrow of the mice [94]. In the same study, Dever et al. used a CRISPR-Cas9 system to correct the (A > T) mutation seen in sickle cell disease, following the standard logical scheme of isolating progenitor cells (in this case, hematopoietic stem and progenitor cells [HSPCs]) taken from patients with the disease, and then performing gene editing. The corrected HSPCs were able to differentiate into erythrocytes expressing β-globin mRNA.

Two more examples of gene editing directed toward correcting sickle cell disease again used a CRISPR-Cas9 system. This time, a ribonucleoprotein complex consisting of the Cas9 protein and an unmodified gRNA was delivered with a single-stranded DNA oligonucleotide to patient-derived HSPC cells by electroporation. Correction efficiencies of up to 25% were reported [95]. The corrected HSPCs were used to restore the expression of wild-type hemoglobin after differentiation into erythroblasts, with the correction being maintained through 16 weeks after injection [95]. A remarkable correction efficiency of up to 67.9% was reported by Li et al. when they used CRISPR-Cas9 with adenovirus, followed by nucleoporation with a single-stranded DNA template, to correct the sickle cell disease mutation in patient-derived iPSCs [96].

As we have seen, the CRISPR-Cas9 system is a popular route for gene editing for treatment of β-hemoglobinopathies. In fact, this system could be deemed the preferred method for editing. A series of studies have demonstrated that CRISPR-Cas9 nucleases are relatively efficient and specific in gene editing in human iPSCs [91,97–99], with advantages over ZFNs and TALENs, even when used to correct a point mutation such as that seen in sickle cell disease [100]. However, this certainly does not invalidate efforts made with the other editing systems, which have been shown to be superior to CRISPR-Cas9 in certain situations.

TALENs have been used to correct separate mutations (the intron 2 mutation IVS2-654C > T and the TCTT deletion) in the *hbb* gene of patient-specific iPSCs, made to enter cells via electroporation. The corrections were achieved with high efficiency: 68% and 40%, respectively [101]. TALENs and CRISPR-Cas9 have been separately employed, combined with a PiggyBac transposon DNA donor, to correct IVS2-654C > T mutations in the globin gene [102]. In this case, contrary to findings reported in the previous paragraph, TALENs performed with higher efficiency than CRISPR-Cas9 (33% versus 12.3%). In addition, the TALENs generated fewer off-target events. Not all such CRISPR-Cas9 applications display off-target effects, however. Fei et al. employed CRISPR/Cas9 combined

with the PiggyBac transposon to correct a −28 (A/G) mutation and TCTT deletion seamlessly in the *hbb* gene in patient-derived iPSCs to restore HBB expression after hematopoietic differentiation [103]. In this case, off-target events were not observed.

In addition to therapeutic applications toward β-hemoglobinopathies, genome editing has been employed to increase red blood cell production for transfusion purposes. The *SH2B3* gene encodes a protein that serves as a negative regulator of cytokine signaling. Naturally occurring dysfunctional variants of the protein can increase the population of red blood cells. CRISPR-Cas9 has been used to delete the *SH2B3* gene in pluripotent stem cells, allowing for the increased production of erythroid cell populations in vitro [104].

Skin

Epidermolysis bullosa dystrophica is a genetic disease caused by mutations within the gene encoding the collagen α-1 (VII) chain (COL7A1). Afflicted patients have fragile skin that is susceptible to minor injury and they easily form blisters and skin erosions.

Efforts targeted to correct this genetic abnormality have been made by codelivering Cas9 and gRNA into iPSCs generated from patients with dominant dystrophic epidermolysis bullosa [105]. After the editing, the iPSCs were differentiated into COL7-secreting keratinocytes and fibroblasts. Another approach was to deliver TALENs, via electroporation, into primary fibroblasts isolated from a patient with the disease in an effort to correct the COL7A1 mutation [106]. The corrected fibroblasts were then driven into iPSCs and placed into the flanks of SCID mice. Results showed that the cells expressed normal COL7A1 protein and that skin-like structures were formed after implantation.

Nerve

As with so many of the applications that have been covered thus far, the CRISPR-Cas9 system has been used to correct genetic mutations associated with neural disorders. The common scheme is to isolate progenitor cells (or to induce isolated cells into iPSCs) from a patient with the affliction and then to correct the mutation with the editing system. The resulting corrected cells are then differentiated and implanted back into the patient. This scheme has been performed on, for example, cells from patients with Alzheimer disease to correct a point mutation in the presenilin 1 gene [107]. It has also been used in conjunction with the PiggyBac transposon system to correct point mutations that result in disruption of tetrahydrobiopterin synthesis or recycling, which affects tyrosine and dopamine levels that are important for proper brain function [108]. The system has also been used to bring about genetic deletions, such as to combat fragile X syndrome, a type of inherited intellectual disability that is caused by an expansion of an area of CGG repeats in the *fragile X mental retardation 1* gene [109].

As mentioned in the Nickases section, Cas9 can be mutated to alter its endonuclease activity, resulting in enzymes known as nickases. Whereas wild-type Cas9 allows for genome editing by introducing targeted double-strand breaks in the genome, mutations of the HNH or RuvC domains in Cas9 can yield nickases that cut only one strand of DNA [110,111]. Some have introduced mutations that stripped Cas9 of all endonuclease activity [112,113]. These mutants, called nuclease-deficient Cas9 (dCas9), have been used for transcriptional repression and activation. CRISPR interference technology uses a fusion of Cas9 with repressor domains to yield effective gene repression. CRISPR-mediated gene activation incorporates a fusion of dCas9 with activation domains to recruit transcription activators in the nuclei of mammalian cells [114].

As an example, consider an application that targeted α-synuclein, which is encoded by the *SNCA* gene and is associated with mediating the pathogenesis of Parkinson disease. Under the mediation of carefully designed gRNA, a dCas9 fused with a hybrid VP64-p65-Rta tripartite activator (dCas9-VPR) [115] was used to obtain upregulation of *SNCA* expression by eightfold compared with iPSC-derived neurons expressing normal α-synuclein levels, whereas dCas9-KRAB served as a transcriptional repressor to downregulate *SNCA* expression by 40% in α-synuclein triplication iPSC-derived neurons [116].

Retina

Retinitis pigmentosa is a group of inherited eye diseases that are caused by mutations within a group of genes. Patients with retinitis pigmentosa have impaired vision caused by the degeneration of photoreceptor cells within the retina. A point mutation (c.3070G > T) in the *retinitis pigmentosa GTPase regulator* gene was corrected using

CRISPR-Cas9 with about 13% efficiency in patient-derived iPSCs, which laid the foundation for autologous iPSC transplantation to treat retinal diseases [117]. In another study, CRISPR-Cas9 was used to disrupt a mutation in the rhodopsin gene in the S334ter rat model of retinitis pigmentosa, halting retinal degeneration and improving optomotor reflexes (a possible indicator of improved visual function) [118].

CLOSING REMARKS

Gene editing involves the cleavage of double-stranded DNA in a targeted fashion. Although restriction enzymes have been used in molecular biology for about half a century, the field of genome editing has arisen relatively recently. The breakthrough in the field of genome editing came with the discovery of endonucleases that have tunable targeting and can be used in living cells. Three major forms of such systems were presented in this chapter: CRISPR/Cas, TALENs, and ZFNs.

For the researcher, the choice of editing system should take into account both the efficiency of a given method and the number of off-target effects generated. A system that produces the desired genetic change in 99.9% of treated cells would not be desirable for clinical use if an off-target insertion, deletion, or base change caused 0.1% of the cells to be transformed into cancer cells. As covered earlier, much work has been performed to generate enzymes that operate with greater specificity in the hope of eliminating off-target effects.

The general scheme used for gene editing for regenerative medicine includes isolating cells from the patient, which will greatly reduce the chances for an immune response to the therapy. The cells may be pluripotent, multipotent, or differentiated, but a common theme is to use or produce stem cells (such as iPSCs). The cellular genomes are then edited and the cells are reimplanted into the host in a pluripotent or differentiated form, with the differentiated form appearing to be more common. Differentiation of the cells before implantation gives the investigator a chance to verify that expression of the edited gene is at a clinically relevant level, whereas implantation of undifferentiated cells may allow for differentiation into multiple cell types in the proportions that the body needs. The implantation itself can occur in the form of the injection of cells into a given organ or tissue, or it could include cellular encapsulation or the embedding of cells into a scaffold construct. These implantation methods are options that are addressed elsewhere in this book.

The regenerative applications presented in this chapter addressed congenital aberrations, or disease states, that were dealt with via gene editing. In the future, it may be possible to regenerate noncongenital tissue defects, such as those arising from injury, by editing cells to be specifically responsive to an engineered repair environment. Editing may be used to create cells that can be used as tools to guide the differentiation of stem cells in vivo, recruit other cells to an area, induce angiogenesis, or proliferate into a tissue that has an enhanced property to aid the host at the organismal level. With new applications and advances appearing in the literature nearly weekly, it is difficult to predict the precise directions that this burgeoning field will take next.

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CHAPTER

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Preclinical Bone Repair Models in Regenerative Medicine

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INTRODUCTION

Bone tissue engineering (BTE) is a promising avenue of research that has the goal of providing novel methods to add to our clinical capabilities for treating difficult segmental and contained skeletal defects. Bone tissue demonstrates the intrinsic properties of regrowth and self-repair, which is a process marked by a complex array of biologic, structural and metabolic functions. The primary building blocks of bone tissue regeneration center on the recapitulation of the natural signaling pathways of bone development and healing, which are modified toward developing modalities to stimulate bone formation in a clinical situation in which the skeletal defect may not heal using currently available methods.

Of the many tissues under investigation, bone repair models continue to be a promising avenue of study primarily owing to the ever-increasing demand for and short supply of bone substitutes [1]. It is estimated that two million bone graft procedures are performed annually worldwide [2]. In the setting of large bony segmental defects that are often caused by polytrauma, pathological fractures, and osteonecrosis, the capacity for the normal process of fracture healing to repair the skeletal defect and restore load-bearing function to the injured bone is often insufficient, and the result is a fracture nonunion. This further marks the importance of preclinical bone repair models and their value for clinical application. This chapter will provide a general focus on the biological processes of preclinical bone repair in vitro and in animal models.

Ethical issues must be addressed when animals are used as preclinical models as part of the testing program. The appropriate conduct of experimentation using live animals is important to progress in the care of both humans and animals. The responsibility for the appropriate use of animals in research is incumbent on the investigators. The three "R's" of animal experimentation are replacement, reduction, and refinement. Replacement is the process of seeking to replace animals in an experimental design by either using an in vitro method or a phylogenetically lower animal whenever possible. The reduction process is constant assessment of the experimental design in an effort to reduce the number of animals by asking whether the desired data can be obtained in a statistically valid manner using fewer animals. Finally, the refinement process is an attempt to improve existing experimental methods to obtain the desired data with reduced ethical costs in terms of any painful or stressful procedures that are done on the animals.

BIOMINERALIZATION AND BONE REGENERATION

The triad of building blocks for bone tissue regeneration are: functionally active osteoblastic cells to secrete new bone matrix (osteogenesis), scaffolds upon which these anchorage-dependent cells attach and from which signaling

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molecules can be delivered to the bone regeneration space (osteoconduction), and growth factors to drive the regeneration process (osteoinduction) [2].

CELL SOURCES

Stem Cells in Bone Tissue Engineering

Stem cells require two fundamentally important characteristics: the ability to self-renew and generate daughter lineages with identical potentialities, as well as the ability to differentiate along one or more lineages [3]. In the field of BTE, commonly used sources of stem cells include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult mesenchymal stem cell (MSCs).

EMBRYONIC STEM CELLS

Human ESCs (hESCs) are derived from embryos generated via in vitro fertilization. Embryos are most often obtained from donors after they are deemed unsuitable for implantation and appropriate consent has been obtained. The preparation of embryos occurs via an antibody application process in which the inner cell mass from the blastocyst stage of the embryo is separated from the trophectoderm. The cells that are derived from the blastocyst inner cell mass are initially plated onto feeder cells. The cells that constitute the inner cell mass then expand to form an hESC cell line [4]. ESCs have two distinctive properties: the ability to proliferate infinitely and the property of pluripotency to differentiate into all three germ layer cell types: ectoderm, endoderm, and mesoderm [5].

Of specific interest in BTE, hESCs can be guided toward differentiation into osteoblast cells in vitro. One method of differentiation was described by Bielby, in which osteogenic cells are derived from three-dimensional (3D) cell spheroids (embryoid bodies [EBs]) [5]. EBs are formed via suspension or hanging drop methods from a single cell suspension. EBs provide the potential for differentiation into precursor cells of all three germ layers. Committed cells in the EB matrix are cultured in monolayer and are induced to osteogenic cells under the presence of exogenous factors (e.g., dexamethasone-ascorbic acid and sodium-β-glycerophosphate). These exogenous factors work in concert to promote an environment conducive to bone regeneration. Dexamethasone stimulates the osteogenic differentiation of precursor cells harvested from multiple tissues. Ascorbic acid facilitates collagen secretion and deposition, and sodium-β-glycerophosphate acts to mineralize the deposited matrix. Working in concert with biochemical exogenous factors, the extracellular matrix (ECM) has an important role in the direction and differentiation of ESCs. Alternatively, undifferentiated ESCs or dispersed EBs are sometimes implanted directly into 3D scaffolds and driven to form multiple tissues for later implantation [6].

Induced Pluripotent Stem Cells

iPSCs are somatic cells reprogrammed to exhibit pluripotent properties. Historically, mouse skin fibroblasts were first reprogrammed to iPSCs by overexpression of a set of four key transcription factors [7]. Subsequently, adult human cells were reprogrammed [7,8]. Systematic reviews of the literature on the osteogenic potential of iPSCs suggest that osteo-induced iPSCs demonstrate an osteogenic capability equal or superior to MSCs [9]. Furthermore, studies demonstrate that cell sources for iPSCs do not appear to affect the osteogenic potential of iPSCs [9]. In their early use, it was noted that iPSCs were met with complications of teratoma formation. In an attempt to address these complications, studies have demonstrated that the addition of resveratrol to the osteogenic medium and irradiation after osteogenic induction reduce teratoma formation in animal models [9].

Mesenchymal Stem Cells

The most common stem cell type used in the BTE field is the MSC [10]. First identified in 1966 by Friedenstein, MSCs were discovered via the isolation of bone and cartilage-forming cells from rat bone marrow cells that had fibroblast-like morphology [11]. MSCs have since been isolated from multiple tissues (liver, fetal blood, and umbilical cord), but the most well-investigated and readily available source of MSCs is the bone marrow. MSCs harvested from different sources may not be identical in their biological characteristics. A comprehensive report on the

proteome and transcriptome profiles of MSCs revealed source-specific markers [12]. Furthermore, differences that exist in colony-forming unit efficiency, surfactome profiles, multilineage differentiation, and paracrine functions may suggest a specific optimal clinical application for that particular MSC cell line [13,14].

MSCs comprise roughly 0.001%−0.1% of the total population of bone marrow nucleated cells. In turn, these can be specifically separated from other nucleated cells by their adherence properties to plastic culture flasks. They can then be expanded in vitro without a loss of phenotype. The process of harvesting MSCs and then exploiting their pluripotent ability to differentiate them into osteoblasts, chondroblasts, myoblasts, and tenocytes is primarily why they are often used in musculoskeletal tissue engineering applications [15]. MSCs have the ability to differentiate into a variety of adult musculoskeletal cells as well as an inherent ability to secrete a variety of cytokines that modulate inflammatory and immune response pathways [16]. The synergistic benefit of these immunomodulatory effects is a reduction in the risk for host rejection. Another beneficial property of MSCs is their ability to be driven down various mesenchymal cellular pathways including chondrogenic, osteogenic, and adipogenic lineages when provided the necessary in vitro or in vivo environments [17] or 3D scaffolds [18]. MSCs are driven toward osteogenic differentiation when stimulated with dexamethasone, ascorbic acid, and sodium-β-glycerophosphate. When appropriately stimulated, MSCs upregulate alkaline phosphatase, osteocalcin, and the expression of osteopontin, and they direct calcium deposition within the ECM. In vivo preclinical BTE models also benefit from the use of MSCs, in that the MSCs have been shown to facilitate bone repair when they have anchored onto artificial matrices such as hydroxyapatite scaffolds [19].

SCAFFOLDS

BTE scaffolds allow for the adherence of osteogenic cells and serve as an appropriate microenvironment to permit those cells to secrete osteoid, the matrix of newly formed bone tissue. Scaffolds are often subdivided into three classes based on their material composition: polymers, metals, and bioceramics. Scaffolds may contain more than one of these three material types. Further stratification is delineated by the derivation of the material (i.e., natural versus synthetic) and their ability to undergo degradation (i.e., resorbable versus nonresorbable).

The benefits of naturally derived scaffolds (i.e., collagens, fibrin, elastin, alginate, hyaluronic acid) are their resorbable qualities in vivo as well as their intrinsic biocompatibility and minimal adverse immunological properties. Synthetic scaffolds have the benefit of often being able to be fabricated with a wider range of degradation rates and mechanical properties compared with their natural counterparts. This ability is based on their polymeric composition, the copolymer ratio, and the interactions of their polymeric side chains. The choice of the synthetic polymer's chemical composition and 3D configuration often determines its cell adhesion properties and ability to incorporate and deliver bioactive molecules in a controlled fashion [20].

A novel arena within scaffold design has been the adoption of additive manufacturing technologies (3D printing) in scaffold fabrication. The term "3D printing" describes a group of additive manufacturing technologies that are collectively referred to as solid free-form fabrication. These include laser stereolithography, fiber electrospinning, fused deposition modeling, fiber extrusion from the melt, and injection molding [21]. The benefits of 3D printing are conferred by its immense flexibility in fabricating scaffolds of varying structural complexity. This process allows for a great deal of control over the construct architecture and flexibility in scaling up fabrication, and it has the added benefit of technically precise reproducibility, which is sometimes lacking in subtractive fabrication techniques (e.g., milling).

Biochemical Signaling: Growth Factors and Cell Signals

BTE cell signals are environmental factors that directly or indirectly influence the regeneration of skeletal tissues. The importance of appropriate cell signals and growth factors cannot be overstated in terms of their importance for bone formation. Common growth factors of particular importance in bone regeneration include bone morphogenic protein (BMP)-2, BMP-7 (also called osteogenic protein 1), fibroblast growth factor, platelet derived growth factor, parathyroid hormone (PTH), PTH-releasing peptide, transforming growth factor-β3, vascular endothelial growth factor, and the Wnt proteins [22]. The interplay between signaling molecules and growth factors is complex and multivariable. Factors critical to cell signaling include the spatiotemporal release of growth factors and their bioactivity. The delivery of biochemical signaling cues is generally categorized as unbound, bound within the implant with a designed controlled delivery, coated on the implant surface, or coded within the cells via gene delivery mechanisms [23,24]. Unbound delivery systems are marked by the rapid efflux of the growth factors followed by rapid clearance from the microenvironment. Bound delivery systems allow the advantage of controlled or specific variable release of the biochemical signal over time. Generally speaking, bound delivery

systems are more applicable for the requirements of BTE. Hydrogels are the most commonly investigated polymer for cell encapsulation and the in situ delivery of biochemical cues [22]. To mimic the function performed by the ECM, bioactive hydrogels containing protease sensitive sites, cell adhesion molecules such as Arg-Gly-Asp–containing peptides, and/or biological cues in the form of growth factors, inorganic minerals, or drugs have also been developed [25,26].

Of the many growth and differentiation factors in BTE, BMPs possess the unique ability to stimulate the differentiation of mesenchymal precursor cells to chondrocytes and osteoblasts; this in turn allows for the induction of new bone at ectopic and orthotopic sites. Marshall Urist discovered that demineralized bone matrix induced ectopic bone formation in subcutaneous and intramuscular pockets in rodents [27]. Further investigation over the next several decades led to the isolation of BMPs as the causative factor in this induction of the bone formation cascade [28]. Clinically, BMPs are often used at the sites of intended bone regeneration in skeletal defects with the goal of osteoblastic cell proliferation and differentiation. The delivery of BMPs can be either direct application to the intended site of bone regeneration or via controlled release. There are several drug delivery systems under investigation that allow for measured controlled delivery. BMPs as well as other proteins may be encapsulated in poly(D,L-lactic-*co*-glycolic acid) microspheres [29] or embedded into collagen carriers [30]. Among other delivery systems, these allow for the temporal and spatial release of growth factors and cell signals in a controlled fashion.

PRECLINICAL MODELS OF BONE TISSUE REGENERATION

In Vitro Preclinical Models

In vitro studies offer the advantage of focused manipulation of specific biomaterials in well-controlled experiments. In targeting specific cascades, investigators are more capable of understanding specific variable responses in a closed environment with the goal of predicting future responses in preclinical animal models and in subsequent translational first-in-human applications. In vitro studies afford the opportunity to simulate complex interactions among cells, scaffolds, and growth factors in relatively controlled environments. Bone-forming cells (osteoblasts) are often procured from three primary origins:

- pluripotent stem cells that differentiate into osteoblasts [31],
- primary osteoblasts and osteocytes from different species [32,33], and
- immortalized cell lines [34,35].

To maintain translational validity, bone in vitro models must incorporate the classic triad of cells, scaffolds, and growth factors in a fashion that closely mimics both the biochemical and biomechanical bone formation characteristics that are seen in vivo. The bone matrix is composed of cells surrounded by an ECM made up of an inorganic phase that is approximately 70 wt% hydroxyapatite and an organic phase that is approximately 30 wt% type I collagen and other noncollagenous proteins [36,37]. Biomechanical studies have demonstrated that greater than 90% of bone cells within the ECM are osteocytes that sense and transduce mechanical forces exerted on the bone, in turn governing the rates of resorption and deposition that occur during bone remodeling [38].

Historically, the study of the interplay between bone physiological and pathological processes has been performed on 2D plastic plates. Although great strides in understanding bone formation, bone resorption, and bone remodeling have been made via this medium, the 2D cell cultures have been shown to mimic the 3D microenvironment of native bone and the interactions between the bone cells and the ECM incompletely [39]. In vitro studies provide great insight into the translational validity of a model, but the biomechanical environment in vivo is much more complex and the stresses exerted on the bone dictate its resorption and deposition rates. This complex interplay between the microscopic cellular level and the macroscopic mechanical level are greatly important in BTE, because understanding these interactions is critical to the success of in vivo BTE preclinical models.

In Vivo Preclinical Models

The benefit of in vivo models rests in the ability to assess biomaterials in more complex tissue environments that have variable loading conditions. Among the limitations of BTE animal models is the interspecies variation of bone tissue. It has been well-illustrated that bone composition, density, and mechanical properties of various animal models (i.e., cow, sheep, pig, dog, chicken, and rat) often differ from those properties in human bone [40].

There are a host of factors to be considered when selecting a specific animal model for in vivo preclinical studies. Chief among these is that the animal model demonstrates physiological and pathophysiological processes that mimic those in humans. The attributes of a "good" animal model are that in addition to being similar to those in humans, as mentioned earlier, the animal's anatomy and physiology are suitable for the requirements of the study design. The animal chosen should be economical and available. The animal management and care issues must have been considered and are optimal for the chosen study (e.g., housing requirements and ease of handling).

Selection Considerations Based on Animal Species

Animal species that are under consideration for in vivo preclinical studies include mice, rats, rabbits, sheep, goats, pigs, dogs, cats, and nonhuman primates. There are pros and cons to the use of any of these animals. Mice and rats are readily available and inexpensive and have minimal housing requirements. On the negative side, their small size is an issue in some experiments, and their life span postskeletal maturity places a time limit on the study. Human clinical conditions that have been modeled by rats and mice include heterotopic bone formation, trabecular bone defects, segmental bone defects, and spinal cord injury. Given their size, robustness, and cost efficiency, rodents are particularly useful in preclinical studies in assessing biomaterials as bone substitutes and are generally regarded as the prime model for in vivo testing of the regeneration of bone tissue [41]. Disadvantages, in addition to those listed earlier, include the lack of Haversian-type remodeling in the rodent bone cortex and thin, fragile cortices in the long bones [42]. The rat, which is the most often used animal model, has been shown to have the most significant difference from human bone compared with other animal models [43]. Rodents are primarily useful for the surgical implantation stage of substitute bone materials. For example, β-tricalcium phosphate (β-TCP), calcium phosphate, and collagen experiments have been commonly conducted in rodents. A prime example of applications of rodent bone defect models is exemplified by investigations of the biocompatibility of highly purified β-TCP bone graft substitutes using a rat femoral defect model. The study demonstrated that purified β-TCP was both biocompatible and resorbable [44]. In another study, investigators successfully used small animal rodent models to establish a 4-mm-diameter calvarial critical-sized defect model in mice. This model was successful in the analysis of the in vivo osteoconductive and osteoinductive abilities of bone substitute materials [45].

Similar to mice and rats, rabbits are also readily available and inexpensive, and have minimal housing requirements. They are larger than mice and rats but are still considered a small animal model. Rabbit models rank as the most commonly employed models in musculoskeletal research [46]. Applications that have used rabbit models include calvarial critical-sized bone defects, posterolateral spine fusion, and cartilage regeneration. Rabbits have similarities to humans in bone mineral density and fracture toughness of middiaphyseal bone.

Large animal models allow for the assessment of a larger volume of bone regeneration and repair over a longer time frame than is possible in mice, rats, and rabbits. Large animal models permit the assessment of bone remodeling and implant integration in a manner that better mimics the biomechanics and loading characteristics seen in humans. Large animal models that are often used in musculoskeletal investigations include sheep, goats, pigs, dogs, cats, and nonhuman primates. The use of sheep, goats, and pigs provides an animal model in which bones and joints are more similar to their counterparts in humans than are those in the small animal models discussed previously. Sheep, goats, and pigs have good availability and can serve as an alternative to dogs in some applications. Dogs and cats are companion animals, which often causes their use to receive greater scrutiny. Negative issues associated with the use of sheep, goats, and pigs include the increased cost, housing requirements, and the need for a formal operating room setup to perform surgery on them. Applications for which sheep, goats, and pigs have been used include radius nonunion (and other bone healing or bone defect applications), femoral head osteonecrosis, anterior cruciate ligament reconstruction, and meniscal repair.

Dogs and cats share the same pros and cons as sheep, goats, and pigs. In addition, as mentioned earlier, their status as companion animals often attracts greater scrutiny in their use in experimental designs that include surgical procedures. Several applications that have used dogs and cats as experimental animals include many of the same applications for which sheep, goats, and pigs were used: radial nonunions, tibial defects, other fracture healing or bone defect models, femoral head osteonecrosis, and craniomandibular reconstruction. In addition, dogs and cats have been used in surgical studies for total joint arthroplasty, spinal cord injury, and distal radius osteosarcoma.

Nonhuman primates have an anatomy and physiology that more closely parallel those of humans than any of the other animal models discussed here. They are not readily available, they are expensive, and they have the highest housing requirements than any of the other animals. The scrutiny that they receive from the Institutional Animal

Care and Use Committee when their use is requested is even higher than that of the companion animals. Applications in which nonhuman primates have been used include osteoporosis, bone healing, fracture nonunions, prosthetic implant studies, spinal fusion, and organ transplantation studies.

Surgical studies using animals are essential for the analysis of novel treatments for both humans and animals. However, we must treat them humanely, take whatever measures are necessary to control their surgical pain, and constantly seek to apply "the three R's" of replacement, reduction, and refinement to obtain the data that we need, and simultaneously try to do so while using increasingly fewer animals.

CONCLUSIONS

No amount or type of preclinical (in vitro and animal) studies can predict with certainty the implant behavior in humans. The first in-human use must consider clinical equipoise: the anticipated balance between potential benefit and potential risk in the human study subject who receives the implant. Consider the regulatory implications of potential preclinical experimental pathways, because different but feasible may pose different regulatory burdens. Plan to speak with US Food and Drug Administration colleagues early and often as the various potential preclinical paths are being considered. Remember that animal models are an essential but insufficient component of the preclinical evaluation for new medical products. The choice of model depends on several factors, including the biologic and structural goal of the study, the applicability of the model to the human condition under evaluation, the cost and technical feasibility of the chosen model, and historical experience with the model. Eventually, the safety and efficacy of new treatment modalities will be determined by well-controlled studies in humans, and postevaluation use in the general population of patients who have the condition to be treated. Be certain that clinicians who care for patients with the disease in question are members of the planning and execution of the preclinical study team. The ultimate determination as to whether your product will become the standard of care for the surgical care of the disease condition under consideration will be made one patient at a time, within the physician–patient relationship as the treating doctor and the patient discuss the various treatment options available to them to treat the patient's clinical problem.

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CHAPTER

44

Body-on-a-Chip: Regenerative Medicine for Personalized Medicine

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INTRODUCTION

Current drug development strategies do not include adequate models to predict drug efficacy or safety in humans. There is a considerable need for the high-fidelity in vitro representation of integrated human physiology to test both the beneficial and potential detrimental effects of drug candidate compounds in the body [1,2]. Animals (rodents, in particular) have been the reference standard of scientific experimentation for centuries. These animal models have served as the cornerstone of research in cell biology, pathobiology, molecular biology, and many other biomedical research fields [3]. Two-dimensional (2D) cell lines and animal models have been used extensively to determine the toxicity of new drugs before the initiation of human clinical trials. It has become clear that these models have significant limitations owing to phenotypic differences in physiology compared with humans. This is especially critical for the assessment of toxic side effects of drugs that might target the liver, heart, and other organs because of the differences in enzymatic expression profiles among humans, rodents, and cell lines, which often results in significant differences in drug metabolism, efficacy, and toxicity. In vitro drug screening platforms use human cells; however, by definition, the genotype of cell lines is altered from the natural state, and primary cells change phenotype after removal from native tissue. These phenotype changes in culture result from the failure of traditional 2D systems to recapitulate several aspects of the native 3D cellular microenvironment [4,5]. As a result, 2D cultures exert selective pressures on cells, significantly altering their phenotype as they adapt to their new conditions. Drug diffusion kinetics are not modeled accurately in 2D tissue culture, drug doses effective in 2D are often ineffective when scaled to patients, and the lack of cell−cell/cell−matrix interactions in 2D often lead to loss of cell function [4,6,7].

In contrast, advances in tissue engineering, biomaterial development, and microfluidics and electronics have resulted in the successful fabrication of multicellular human tissue equivalents and microorgans (organoids) that demonstrate many of the functional properties of normal human tissue and organs. For example, liver organoids exhibit normal metabolic activity, skeletal and cardiac muscle constructs contract in a physiologically normal manner, lung organoids "breath," and gut/vessel/brain microvasculature constructs maintain normal barrier functionality [8,9].

"Body-on-a-chip" devices that recapitulate 3D tissue architectures and physiological fluid flow conditions are more effective at supporting normal cell function than static 2D culture [10]. These engineered platforms can include sophisticated hardware systems, potential for scale-up, capacity for high throughput, and user control over physical factors such as fluid shear stress and mechanical deformations. Microfabrication techniques based on a variety of nanotechnologies have resulted in the development of microscale fluidic systems with predictable fluid dynamics

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throughout the entire fluid circuit [11]. Tissues and organoids can be immobilized within platform microreactors using sophisticated hydrogel biomaterials, providing a proper microenvironment and allowing for long-term perfusion. A variety of iterations on these basic concepts are in use by laboratories around the world [12–14]. In addition, a variety of on-chip disease models have been investigated [13]. The most pressing challenge in advancing the body-on-a-chip field is to combine multiple organs within a common microfluidic circuit, to model an entire human, on-chip. Such a system would represent the pinnacle of in vitro platforms for modeling integrated human physiology. Ideally, as in the human body, such a system would recapitulate the interdependent and synergistic functions of all tissues and organs within a cell culture or body-on-a-chip platform. The microfluidic circuit connecting organoid microreactor chambers allows for fluid flow across each organoid type in a sequence that mimics blood flow throughout the human body [8]. Compound metabolites and other secreted factors would likewise be transported to downstream organoid types in a physiologically relevant sequence. As such, these advanced body-on-a-chip platforms would be ideal for testing newly developed drugs and assessing potential toxic side effects in human tissues and organs. Furthermore, the body-on-a-chip platform would offer tremendous benefits for pharmacological studies aimed at determining the specific effects and toxic levels for newly developed drugs, allowing for the better prediction of appropriate doses for human trials. In this chapter, we highlight a variety of organoid-on-a-chip systems for applications such as drug screening and disease modeling and look to the future of multiorganoid body-on-a-chip systems and applications in personalized precision medicine.

ADVANCE OF IN VITRO ORGANOID DEVELOPMENT: PROGRESSION FROM TWO-DIMENSIONAL TO THREE-DIMENSIONAL MODELS

The development of novel drugs that are safe and effective in humans has been significantly hampered owing to the inability to model human cell phenotype, function, and intercellular signaling accurately in vitro. Animal models used extensively in preclinical drug studies are traditionally regarded to be the reference standard for drug testing. However, animal models do not reflect human drug metabolism accurately; thus, animals are often not predictive of results in humans. The second type of conventional model system, in vitro 2D culture, fails to recapitulate many aspects of the 3D cellular microenvironment, leading to poor support for cell viability and cellular function [4,15]. In addition, drug diffusion kinetics are completely nonphysiological in traditional cell culture and drug doses that are effective in 2D are often ineffective when scaled to patients [6,7]. Cells grown on tissue culture plastic experience several properties in their environment that are inconsistent with the tissue from which the cells were originally isolated. These include surface microtopography, stiffness, oxygen tension, mechanical loading, biochemical composition, and most important, a 2D rather than 3D architecture. These unnatural characteristics can significantly alter the phenotypic properties of cells, because they are forced to adapt to these new conditions.

The functional differences between 2D cultures and 3D constructs have begun to be appreciated. A myriad of studies have demonstrated these differences across a variety of cell types. 3D systems consistently outperform 2D cultures in many aspects including accurately representing in vivo function and demonstrating a physiologically normal response to drugs and toxins [16]. The current drug development pipeline (Fig. 44.1A) has not yet evolved to include newer 3D cell culture technology, which results in countless discrepancies between in vitro drug screening outcomes and later performance in patients, during or even after clinical trials [17]. As an example, our team demonstrated that metastatic colon carcinoma cells adopted an epithelial appearance in 2D tissue culture. However, when transitioned to a 3D organoid form factor, the cancer cells "switched" to a morphology that resembled mesenchymal metastatic cells, which were much more representative of malignant tumor cells in vivo [18]. These kinds of documented benefits of 3D cell culture beg the question: Why are 2D cell cultures still being employed in drug development and toxicology screening?

Fortunately, tissue engineering technologies have evolved to the point that microengineered tissue constructs can better mimic the structure, cellular heterogeneity, and function of in vivo tissue. These organ models can often be maintained in viable states for longer periods and are designed to preserve the functional properties of native tissues. They can also recapitulate the microenvironmental roles of cell–cell, cell–extracellular matrix (ECM), and mechanical interactions that cells experience inside tissues. Oxygenation can be a concern in 3D tissue models. If they are too large in diameter, an oxygen gradient develops across the organoid that can lead to phenotypic changes and potentially a necrotic core. However, oxygen gradients exist in vivo. As such, as long these gradients are taken into consideration and controlled, either by limiting the size of the organoids or by creating perfusable channels within the construct, maintaining an oxygen gradient may actually provide a better representation of native tissue. Overall, these relatively new 3D model systems are greatly superior to their 2D predecessors for drug and toxicology testing.

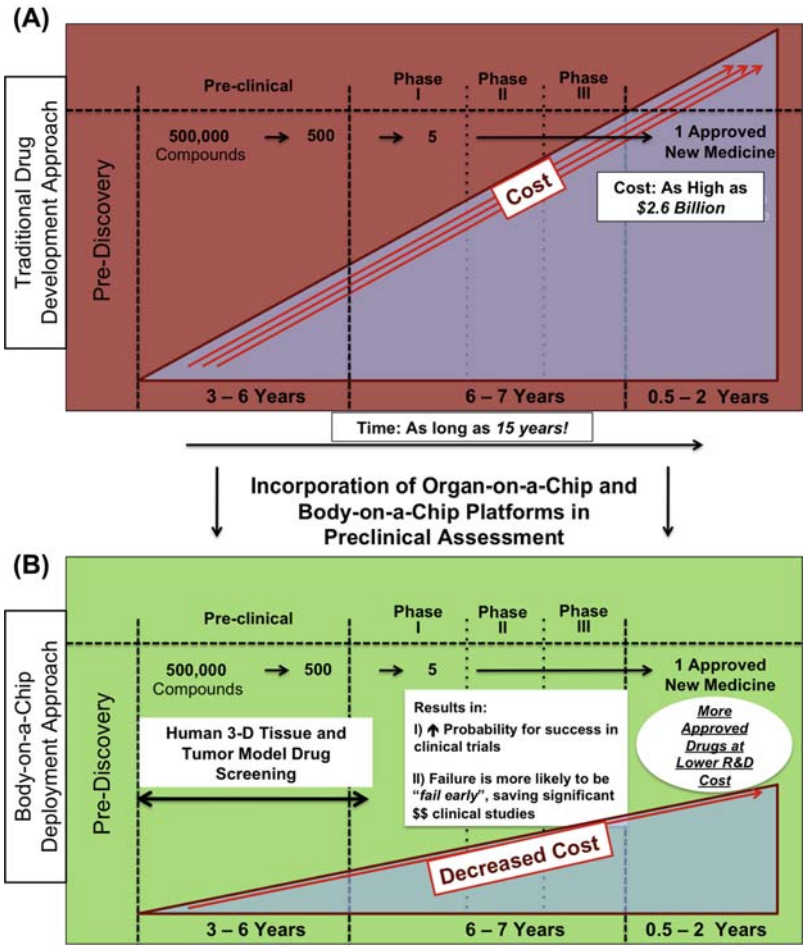

FIGURE 44.1 **Potential improvements in the drug development pipeline as a result of deployment of organ-on-a-chip and body-on-a-chip technologies into pharmaceutical research and development.** (A) The current drug development pipeline requires many years and multiple billions of dollars to bring a drug to market. (B) Plugging in human-based, biofabricated on-a-chip platforms into preclinical stages can potentially drastically improve the efficiency of the drug development pipeline. *R&D*, research and development.

Fortunately, the general concept of performing research using 3D versus 2D cultures has gained significant traction. However, hurdles and challenges remain. 2D cell culture is an established practice that will certainly remain a widely used tool for many years. This is primarily because it is easy and inexpensive compared with 3D culture systems. Implementing 3D systems in a laboratory can be complicated, requiring the mastery of new technologies including biomaterial development and biofabrication techniques. Furthermore, once 3D culture technologies have been established, processes regarded as trivial in 2D culture, such as cell harvesting and cell passaging, can be difficult and in some cases impossible without harming the cells. For example, if cells are cultured within a 3D hydrogel construct, one must effectively dissolve the matrix to isolate or harvest the cells. Some biomaterials support cell retrieval by building specific features into the material [19], but most do not; instead, they require enzymatic dissolution that in some cases can influence cell viability or phenotype. Also, most cell imaging techniques were developed for 2D cell cultures, environments in which cells exist in a narrow focal plane. In 3D, cells reside in many focal planes. Consequently, high-quality imaging in 3D may be obtained only by confocal or macroconfocal microscopy, requiring expensive equipment to which many laboratories do not have access. In addition, there are a variety of assays that can be significantly more difficult to perform on 3D models or that require significant modification for adapting to 3D models. Finally, some body-on-chip device materials (polydimethylsiloxane, for example) are prone to fouling and drug and protein adsorption. However, new materials for device hardware are being developed to solve this problem.

All things considered, it is generally understood that 3D or dynamic on-a-chip platforms outperform static 2D systems in modeling normal human physiology [16]. As a result, these more capable 3D platforms have an immense potential to influence the drug development pipeline positively, decreasing development costs and increasing success rates of drug candidates in clinical trials (Fig. 44.1B). Perhaps just as important, these models can be used to identify nonoptimal drug candidates early before human trials are initiated.

ORGAN-ON-A-CHIP TECHNOLOGIES AND THEIR APPLICATIONS

Advances in biotechnology areas such as tissue engineering [20], biomaterials [21], and microbiofabrication [22] have allowed the derivation of new biological systems with massive potential as test platforms. Researchers have developed a wide variety of human-derived in vitro models that can be used to test drugs, toxins, and drug candidates in a more normal physiological environment [14,23–25]. Furthermore, advances in molecular genetics and tissue engineering technologies have enabled the development of 3D models of specific diseases [13,26–28]. Advances in related technologies such as microfabrication and microfluidic technology have improved 3D cell models by supporting cell and organoid culture, fluid flow, high-throughput testing, environmental sampling, and biosensing. These organ-on-a-chip systems can vary widely in design, allowing for the representation of a range of tissue types. Some of these systems have already been implemented in drug discovery [12] and purport to affect the future of medicine significantly. Here, we highlight just a few microengineering technologies and discuss examples of liver-on-a-chip, vessel-on-a-chip, lung-on-a-chip, and cancer-on-a-chip systems. However, there are many variations of these systems, as well as many additional models for virtually any tissue type.

Microengineering and Biofabrication

The cellular content of a body-on-a-chip model is only one piece of the puzzle. Even with a physiologically normal composition of cells, normal cell function and appropriate responses to pharmacological agents are not guaranteed. Another environmental characteristic that must be considered is the interaction of cells with specific ECM proteins. As mentioned, cells receive a lot of information from their immediate local microenvironment that directly influences cell phenotype. Thus, it is critically important to reproduce as many of the components of the microenvironment as possible. Several techniques have been developed to incorporate natural structural and functional components of tissue ECM into cell culture systems.

Micropatterning is the precise placement of proteins within a cell culture substrate. Micropatterning can be accomplished by a variety of methods including (1) microcontact printing, in which a stamp is coated with specific ECM components and is pressed against a solid substrate to create a specific pattern; (2) photopatterning using UV light projected through a photomask to catalyze the adherence of ECM proteins, or compounds that bind ECM proteins to a cell substrate in a predetermined pattern; and (3) laser patterning, in which laser light is used to mediate protein binding to a substrate in any desired pattern with very high resolution [29].

Many bioregulatory components of the ECM can be distributed in a controlled manner by using micropatterning techniques. For instance, depositing islands of ECM cell adhesion proteins that restrict cell spreading will induce apoptosis in bovine adrenal capillary cells while maintaining the differentiation state of epidermal keratinocytes. Conversely, micropatterning of ECM proteins that induce cell spreading promoted proliferation of both the bovine adrenal capillary cells and epidermal keratinocytes. These experiments demonstrate a clear link among the local ECM composition of a cell substrate, cell cycle, and differentiation [30,31]. This notion is further highlighted by another study involving micropatterning. Mesenchymal stem cells grown on small micropatterned patches that restrict cell spreading promoted differentiation toward adipogenic lineages, whereas micropatterning of factors that induce cell spreading promoted differentiation toward osteogenic lineages. These studies also showed that modulating cell shape was sufficient to induce the expression of signaling proteins Rac1 and N-cadherin, which have an important role in cell lineage specification [32,33]. Micropatterning represents a powerful tool for precisely controlling the protein composition of the cellular microenvironment within a cell culture substrate. By modulating the biochemical and geometric properties of the microenvironment, global cell phenotype and cell viability may be greatly influenced. Micropatterning has a wide variety of applications in advanced in vitro models and will become increasingly used to tune physiologic output from these systems.

Whereas micropatterning technologies are generally used to control the cellular microenvironment in 2D culture systems, bioprinting provides a method for doing the same in a 3D space. Bioprinting involves the layer-by-layer deposition of structural material, cells, and bioregulatory factors in a controlled manner. Bioprinting technologies enable the fabrication of complex cellularized 3D constructs that may include components of the ECM, including both intrinsic and bound bioregulatory factors that modulate cell organization and function within a 3D space.

Bioprinting is highly customizable across a wide range of resolutions and biochemical or physical characteristics. Applications requiring a more rigid structure can be printed using biomaterials with high mechanical stiffness. For stiffnesses beyond that which may be directly printed, methods have been developed for cross-linking structural components of the biomaterial subsequent to bioprinting. For example, dental implants have been bioprinted

from polycaprolactone and hydroxyapatite [34]. On the other end of the stiffness spectrum, soft tissues such as vascular grafts have been printed using very low stiffness poly(ethylene glycol) hydrogels [35]. Because of the increasing resolution and speed of bioprinters, the structures that may be fabricated are becoming highly complex. The ability to precisely reproduce the structural and biochemical microarchitecture of tissues will most certainly result in more physiologically normal cellular function in bioprinted constructs.

Several biological constructs have been fabricated using current bioprinting technology. Liver organoids have been generated using microextrusion bioprinting technology that includes several liver cell types suspended in a supportive hydrogel. These constructs demonstrated exceptionally high levels of liver tissue function and maintained viability in the long term, which makes them ideal candidates for tissue-on-a-chip applications [36]. Skin substitutes have also been created using a laser-based bioprinting technology, which allowed the precise placement of cells associated with specific layers of the skin. The resulting skin constructs were implanted into rodent wound models and demonstrated robust neovascularization, differentiation of mature keratinocytes, and the generation of a normal dermal basal lamina, all hallmarks of native skin [37].

3D printing may still be considered a nascent field. Logistical obstacles continue to limit applications in whole-organ biofabrication. However, the speed, reproducibility, and scalability of bioprinting make it an ideal complement to body-on-a-chip modeling. 3D printing can be used to generate an industrial-scale volume of biological constructs with low run-to-run variability, with the complex structure required for high-function tissue organoid models.

Liver-on-a-Chip

Early tissue/organoid-on-a-chip devices were geometrically designed to drive cell aggregation, thereby creating multicellular organoids. For example, devices were designed with microwells with a convergent geometry that terminated in a cell substrate of some type. Based on the microwell design, liver-derived cell lines could be formed into either spheroid or cylindrical constructs in a highly controlled manner. These 3D constructs maintained much better cellular function than did 2D controls [38,39]. In another example, spheroids were created from a cell line using an array of channels connecting inverted, pyramid-shaped microwells, allowing for the delivery of cells and test compound to multiple chambers simultaneously. This integration of microfluidics with an array of microreactors greatly increased the throughput potential for drug screening [40].

Liver-on-a-chip devices have become much more complex. They often employ controlled fluid flow to address nutrient circulation, drug or toxin administration, sample collection, and the integration of liver organoids with other tissue types. The latter will be discussed in detail later in this chapter. In one such liver-on-a-chip, hydrogels were used to encapsulate HepG2 cells with National Institutes of Health (NIH)-3T3 fibroblasts. These arrays of 3D organoids had increased liver function compared with 2D controls and produced an appropriate toxic response to acetyl-para-aminophenol (acetaminophen [APAP]) in a drug screening experiment [41]. Our group employed a versatile photopolymerizable hyaluronic acid biopolymer system for in situ photopatterning of HepG2 cells to generate 3D liver constructs. The constructs were formed in parallel channel fluidic devices that were fabricated by soft lithography and molded polydimethylsiloxane. This system was used for toxicity screening by administering multiple alcohol concentrations within each chip. As expected, alcohol administration resulted in a dose-dependent decrease in viability and cellular function [14]. Efforts within our group are focused on miniaturizing this and other systems to increase throughput further. Miniaturization and microfabrication approaches can be employed to generate more intricate biological microarchitecture such as liver sinusoids. Precise seeding and layering of hepatocytes and endothelial cells within microfluidic circuits can be used to generate structures with the resolution required to produce sinusoid-like models [42]. Another approach to generating biologically relevant microarchitecture involves mating synthetic and biological components. As an example, semiporous membrane to separate two adjacent chambers may be used to partition human hepatocytes from sinusoidal endothelial cells. Such a design was shown to generate higher albumin and urea production compared with traditional hepatocyte cultures; it demonstrates another strategy for recapitulating normal microarchitecture to increase cell function [43].

Vessel-on-a-Chip

The term “microfluidics” carries with it the assumption of controlled fluid routing. Thus, microfluidic devices are effective for modeling vascular networks. Moreover, because drugs are generally introduced directly to the bloodstream or enter the bloodstream shortly after oral or airway introduction, fluidic systems that mimic the role of the

vasculature contribution to pharmacokinetics represent a beneficial component for any drug screening technology. A substantial number of vascular-like fluidic devices have been developed, including both straight channels devices [44,45] and fluidic devices with more complex, branching features [46,47]. One major role of the vascular system, beyond transporting nutrients and oxygen among tissues, is to transport drugs and other molecules to sites throughout the body, where they pass through the endothelium into adjacent tissues. Many microfluidic systems have been designed to model the transendothelial delivery of test compounds to a target tissue. As an example, a device was developed that includes two perpendicular channels that cross at a single point. A semipermeable membrane colonized with an endothelial cell monolayer was positioned at the point where the two channels cross. Fluorescently labeled albumin was introduced into one channel and transport through the endothelial monolayer was quantified by laser excitation of the fluorophore in the other channel [48]. In another example, an endothelialized construct was designed with a mechanism to control shear stress experienced by the endothelial cells. The device was used to determine the effect of fluid shear on nanoparticle translocation across the endothelial monolayer. These studies were intended to define the ability of flow and shear stress to model different types of vasculature in terms of the contribution to pharmacokinetics and drug biodistribution [49]. Other microfluidic devices with integrated vasculature have been developed to determine how the atomic structure of drugs and nanoparticles can influence the rate of translocation across an endothelium [50]. The integration of vascular function in organ-on-a-chip microfluidic design shows great promise in providing more accurate modeling of drug pharmacology in next-generation in vitro cell platforms.

Lung-on-a-Chip

The lungs, which represent a fluid−air interface between the aqueous in vivo environment and the extracorporeal environment, serve as a common port of entry for drugs, toxins, pathogens, and other xenobiotic compounds. Accurate modeling of the lung in organ-on-a-chip systems is likely important for relevant modeling of the effects of agents that enter the circulation through the alveoli. Significant advancements have been made in the on-chip modeling of lung tissue [51]. Many of these lung constructs consist of lung epithelial cells and endothelial cells situated on opposing surfaces of a semipermeable membrane. The cellularized membrane forms a barrier that can model the transport of aerosols or vapors from the gaseous alveolar compartment into the aqueous circulatory compartment. Contact of the alveolar epithelial cells with air in the alveolar compartment has the added advantage of promoting normal cellular function and maintaining the differentiated state. In more complex devices, multiple independent pneumatic channels were incorporated into the design. Cyclic deformation of the pneumatic channel walls paired with controlled shear within the fluid channel promoted exceptional cell morphology and function [52,53]. These advanced models have proven to be valuable for modeling several lung pathologies including inflammation, pulmonary edema, mucus plug rupture, alveolar epithelial cell damage, and advanced drug screening [54−57]. Although planar air−fluid interface models have shown incredible promise for modeling exchange across the alveoli and certain pulmonary disease states, more simple, acinar lung organoids may be sufficient for screening drug toxicity for compounds delivered orally or directly into the circulation.

Heart-on-a-Chip

Models for cardiac tissue are generally straightforward in design. The heart's sole function in the human body is to drive the circulation of blood, and most in vitro cardiac models are designed to model this function. Simple monolayer cultures of human cardiomyocytes will beat spontaneously in culture when grown on Matrigel [58]. Sheets of human cardiomyocytes may be layered to produce 3D cardiac constructs that retain the ability to contract in synchrony [59]. These planar construct designs are sufficient for modeling the heart's beating action but are not ideal for modeling 3D mechanics such as contractile force. 3D cardiac constructs consisting of human cardiomyocytes embedded in collagen I hydrogels molded into ring structures self-organize into circumferentially aligned cell architecture and support physiologically relevant action potential propagation [60]. These types of constructs have been integrated into microfluidic circuits to form dynamic, contractile heart-on-a-chip systems [61−65].

Cancer-on-a-Chip

In addition to modeling normal tissue such as liver and heart, excellent models for tumor tissue have been developed. These models have been integrated into microfluidic platforms to form tumor-on-a-chip devices capable of

modeling both tumor growth and metastasis. The microenvironments of tumors are often more complex than their normal counterparts. The ECM often varies greatly from tumor to tumor, and cells within the tumor display varying degrees of reliance on the tumor stroma. Tumors possess a range of degrees of vascularization and have different structural and regulatory protein compositions within the stroma. The physical and biochemical characteristics of tumors can be monitored and controlled using microfluidic and microfabrication techniques. Tumor microphysiological systems derived from a patient's tumor sample can be used to determine the selection of an effective chemotherapeutic agent and an optimal dose on a patient-by-patient basis [66,67].

Advancements in tumor-on-a-chip modeling include the development of integrated hardware to monitor the tumor tissue. These include advanced imaging technology and onboard molecular biology assays that provide detailed characterization of tumor behavior, on-chip. The microscale of on-a-chip systems has been shown to influence cell metabolism significantly. This results from the bioavailability of oxygen within these platforms. Studies demonstrated that microfluidic systems provided greater access to oxygen compared with standard 2D culture systems. This increased oxygen level results in increased Krebs cycle activity and decreased expression of hypoxia-regulated factor-1 [68]. Constant perfusion of with oxygenated media provides a better model for a normal tumor microenvironment.

A device was designed with multiple drug gradient mixers and parallel cell culture chambers to facilitate multidose drug screens. This system was paired with a cell-labeling strategy and high content-imaging data collection on-chip [69]. Another tumor model design includes microscale bioreactors that contain hepatocytes, nonparenchymal cells, and breast cancer cells. This system is intended to simulate the hepatic microenvironment. The device contains oxygen sensors, micropumps for controlling nutrient distribution, and real-time sampling capabilities [70]. This device was used to demonstrate that breast tumor cells will spontaneously become dormant when placed within the hepatic niche. This effect was postulated to result from the microenvironmental cytokine profile created by the presence of hepatic cells within the liver bioreactor. Breast cancer has also been studied using a developed system that includes both the ductal and lobular components of breast tissue [71,72]. This system is intended to model the interaction among these two microanatomical compartments during tumor initiation and progression. In another device, HCT-116 human colorectal carcinoma cells and HepG2 hepatoma cells were encapsulated in Matrigel in separate chambers. Myeloblasts were embedded in alginate gels within an additional chamber to simulate bone marrow. Using this platform, the cytotoxic effects of the 5-fluorouracil (5-FU) prodrug tegafur could be determined for each cell type. Interestingly, using 3D tumor organoids, the liver constructs were able to metabolize tegafur to 5-FU, resulting in cell death within the other two tumor organoid types. Tumor models constructed in 2D were unable to metabolize the prodrug to its activated form [73].

Lung tumor models have also been developed in microfluidic devices. Human non−small cell lung cancer in both 2D and 3D organoid configurations were evaluated for sensitivity to several common chemotherapeutic agents [74]. In another example, lung cancer spheroids were formed from cell lines or derived from patient lung tumor biopsies, both with and without the addition of a pericyte population. Each of these constructs was tested for susceptibility to the drug cisplatin. Systems that included the pericyte population demonstrated higher levels of chemoresistance to the anticancer drug, which indicated the importance of considering all cell types within the tumor when developing an organoid design strategy [75]. These examples of tumor-on-a-chip platforms demonstrate the benefits of 3D organoid models and microfluidic technologies for cancer research as well as patient-specific drug and dose selection.

BODY-ON-A-CHIP: MULTIORGAN SYSTEMS AND FUTURE APPLICATIONS

On-a-chip technologies have gained significant momentum. Although these technologies are relatively new, they have shown great promise for applications in research and drug development. However, systems of increased biological complexity have begun to emerge that feature multiple organoids integrated within a single platform [76−79]. These multiorganoid devices [80], sometimes referred to as "body-on-a-chip" systems, have demonstrated greatly increased potential for modeling relevant physiology compared with single organoid systems. Until recently, these multiorganoid platforms were composed of cell lines and animal cells [73,81] More recent systems have begun to use human primary cells or fully differentiated cells derived from stem or progenitor populations. These human platforms have required the development of more advanced cell substrates and microfluidic devices to support primary cell populations. Several notable published studies have demonstrated complex human multitissue systems. In one such system, a four-tissue circuit was developed in a pumpless microfluidic perfusion platform, housing 2D tissue cultures of liver, cardiac, skeletal muscle, and neuronal integrated within

a single microfluidic circuit. This platform was designed as a screening tool to determine cell toxicity in experiments using doxorubicin, atorvastatin, valproic acid, APAP, and *N*-acetyl-*m*-aminophenol [82]. This pumpless concept was also employed in a dual-tissue gut−liver system including transepithelial electrical resistance sensors to monitor gut epithelial barrier function [83]. In another study, a dual-organoid microfluidic platform was developed with integrated intestine, skin, liver, and kidney epithelial barrier tissue. In that system, basic cellular function, appropriate gene expression, and viability were maintained for 28 days [77]. These examples represent important steps toward systems that can mimic complex, multitissue responses and interactions during drug and toxicology testing.

The Importance of Multiorganoid Integration

In vitro models that recapitulate human tissues and model disease accurately are rare. Fewer still contain multiple tissues integrated in series on a single platform. Such models are required for testing drug toxicity and effects at the organism level, because tissues do not exist in isolation in the body. Moreover, for cells within a microphysiological platform to function normally, it is essential that they receive signals and support, such as vascular, neural, metabolic, and hormonal cues from other cell types. With respect to drugs, effects in secondary tissues can be as important as those at the intended target site, particularly if they induce toxicity. If undetected, detrimental secondary effects can lead to the failure of expensive clinical trials or withdrawal from commercial or clinical use after US Food and Drug Administration (FDA) approval. Multiorganoid platforms are also useful for disease modeling. Cancer metastasis, in which malignant tumor cells are able to migrate to and establish tumors at a secondary site, may be modeled in multiorganoid platforms. Although they are useful for many applications, single-organoid models have a limited ability to model these types of physiologically relevant events. Here, we describe several examples of multiorganoid platforms that demonstrate the importance of these systems.

Cancer

As described earlier, cancer metastasis is a disease process that may be modeled only in a multiorganoid system. In developing metastatic potential, certain tumor cells gain the ability to intravasate through endothelium into the bloodstream or lymphatic system. They may then migrate to a distant tissue and extravasate into a secondary tissue site. Few in vitro systems have been developed that employ a multiorganoid approach to model the kinetics of metastasis. One system developed by our team demonstrated that it is possible to recapitulate the metastatic process in vitro. This metastasis-on-a-chip platform was designed to enable tracking of the migrating metastatic tumor cells from a bioengineered colon organoid to a bioengineered liver organoid within a simple recirculating microfluidic device (Fig. 44.2A). It was shown that metastatic colorectal cancer cells were able to migrate out of the colon tumor organoid into the microfluidic circuit and engraft in the downstream liver organoid. Conversely, a nonmetastatic colorectal cancer cell type proliferated at the primary site but never migrated to the liver within the study time frame [84].

Tumor metastasis-on-a-chip platforms, as described previously, may be composed of multiple organoids that enable tumor cells to metastasize from a primary location to a secondary site. On-a-chip devices have also been designed to assess certain discrete aspects of metastasis. For example, one system includes a microfluidic device that can model the process by which multicellular tumor aggregates migrate through both a collagen matrix and an endothelial cell layer [85]. Another device includes an endothelial cell layer that partitions a microfluidic circuit from a chamber that houses a 3D bone construct. This system allows modeling of the extravasation of metastatic tumor cells from the vasculature into bone [86,87]. Other devices include a system to assess the effects of interstitial pressure on cell migration [88] and a system for screening antiangiogenic drugs [89]. These systems illustrate the potential benefits that on-a-chip cancer technologies are capable of delivering. However, there is still a major lack of platforms that model both the primary and metastatic sites, as well as the barriers that separate these locations (i.e., basement membranes, circulation, ECM, and endothelium) in a single platform. By providing circulating flow through a system containing both vasculature and multiple organoids, recapitulating the migration of tumor cells from primary tumor organoids into the microfluidic circulation and engraftment into a downstream target organoid may be accomplished. The results of these systems seem to be well-aligned with what is seen clinically. For example, this system has demonstrated that colorectal cancer cells preferentially engraft into liver organoids, a well-established target tissue for colorectal metastatic tumors [84]. These examples represent several components of the metastatic process that have been modeled in multiorganoid systems; future studies will likely rely on these types of platforms to uncover other factors that influence metastasis.

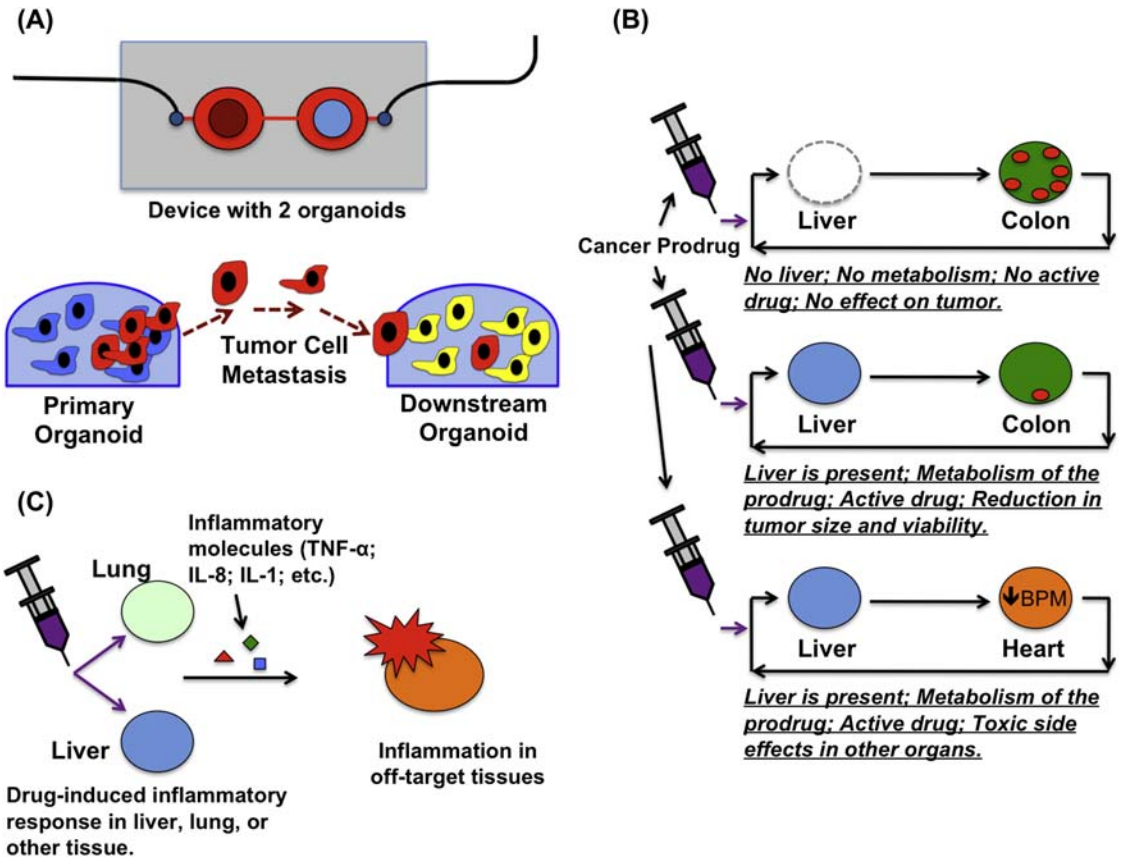

FIGURE 44.2 **Examples of multiorgan interactions that cannot be modeled with single-organoid systems.** (A) Migration and metastasis of tumor cells from one organ or organoid site to another, demonstrated in vitro in a metastasis-on-a-chip device in which colorectal carcinoma metastasizes from the colon to the liver. (B) Reliance of a prodrug therapy such as the anticancer 5-fluorouracil prodrug tegafur on liver metabolism to activate the drug to generate a positive effect by targeting tumor cells successfully. (C) Inflammatory molecules secreted from organs such as the liver and lung upon drug injury can cause detrimental inflammatory responses and cell injury in downstream tissues. *BPM*, beats per minute; *IL*, interleukin; *TNF-α*, tumor necrosis factor- α.

Drug Testing and Toxicology

As has been discussed throughout this chapter, a major area of interest addressed by the body-on-a-chip field is understanding how multiple organs and tissues respond to the administration of particular drugs within an integrated platform. A variety of examples demonstrate this concept. For example, 5-FU is a common chemotherapy agent employed in treating colorectal cancer. Unfortunately, 5-FU can induce a variety of detrimental side effects in patients, including cell damage in the gastrointestinal tract. In an attempt to reduce toxicity, several prodrugs have been developed, such as tegafur. Tegafur and other prodrugs are inactive in the administered form. The prodrug is activated by hepatocytes in the liver to 5-FU that are able to kill tumor cells. Consequently, without including a metabolically active liver organoid in the system, no active drug would be produced and experimental results would be irrelevant. Including functional liver organoids, along with intended target tumor cells and potential tissues that may experience unwanted toxicity, a more complete understanding of the benefits and risks associated with administration of a chemotherapeutic agent may be determined (Fig. 44.2B).

Additional strides are being made toward deploying organoids and organ-on-a-chip technologies in drug and toxicology screening applications. For example, our group demonstrated the use of 3D cardiac organoids in screening for drugs and toxins. We consistently observed expected changes in cardiac beat kinetics in response to these compounds. By employing a minimicroscope with custom-written software to analyze cardiac beating kinetics, the precise determination of beat frequency and magnitude could be recorded [64]. These toxicity screening efforts were expanded to include both cardiac and liver organoids, assessing toxic outcomes from a panel of environmental toxins and a set of drugs that were recalled from the market owing to unanticipated toxicity in human patients (Fig. 44.3). Fig. 44.4 highlights some of these studies, in which the dual-organoid systems were able to model

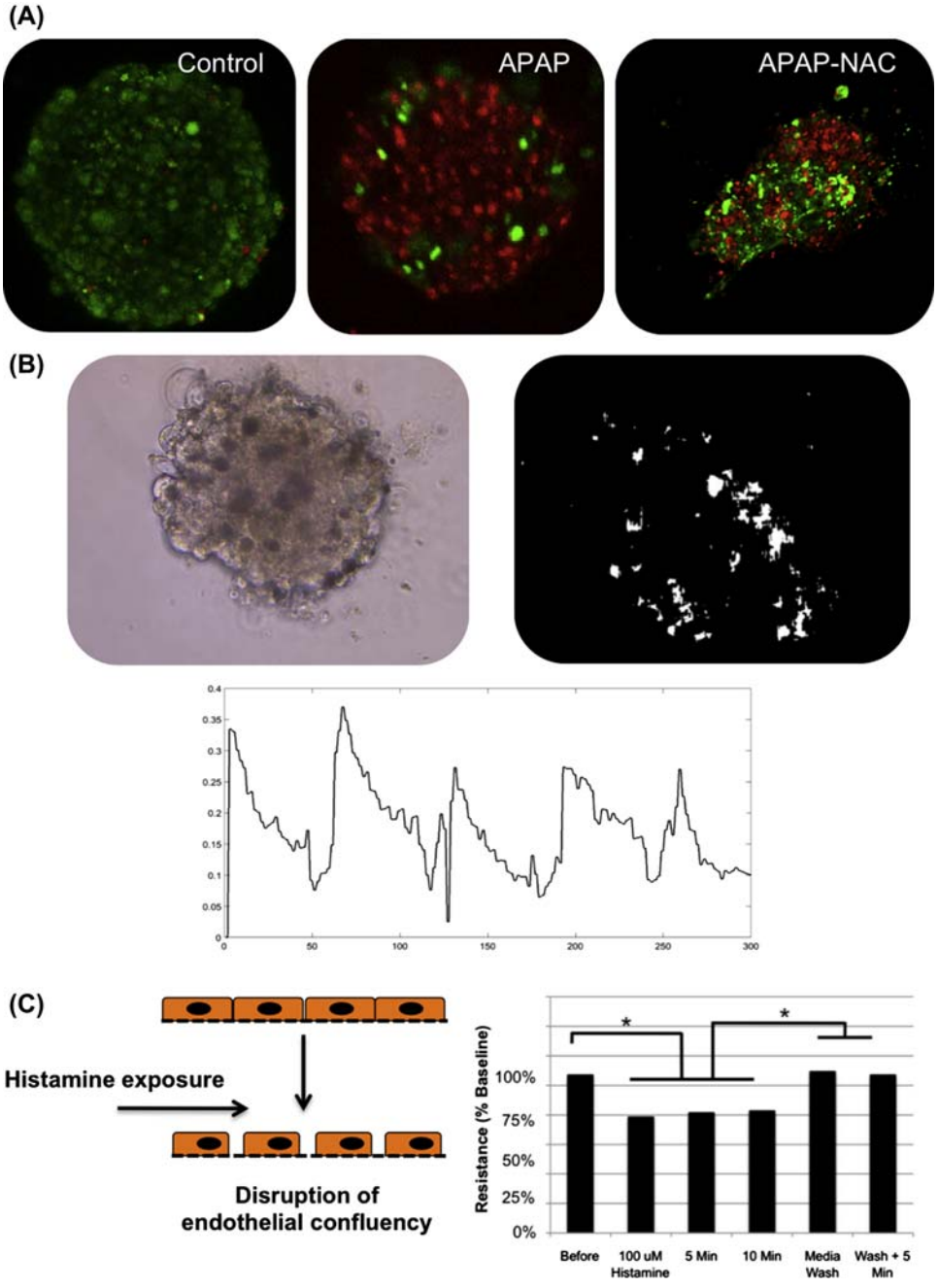

FIGURE 44.3 **Highly functional organoids for a multiorganoid body-on-a-chip platform.** (A) Acetaminophen (toxicity in liver organoids and reduction in toxicity by *N*-acetyl-L-cysteine. (B) Cardiac organoids remain viable long-term and support transport of fluorescent dyes (lucifer yellow [*yellow stain*] and fluorescein [*green stain*]) through interconnected ion channels suggesting high levels of cell–cell communication. (C) Beating analysis of cardiac organoids: An onboard camera captures video of beating organoids, after which beating rates are calculated by quantifying pixel movement, generating beat plots. (D) Vascular endothelium devices respond to changes in endothelium integrity as measured by a transendothelium electrical resistance sensor. Organoid diameter - 250 μm.

these toxicities. In addition, the integration of different tissue organoids into a singular system allows for screening studies that can identify unanticipated toxicities (Fig. 44.2C) [90].

Additional Disease Modeling

Research into human pathologies other than cancer may also benefit from the capabilities of multiorganoid systems. Many drugs and that are known to cause inflammatory responses. For example, large doses of the common analgesic APAP (Tylenol) causes significant inflammation and toxicity in the liver. Other drugs, including

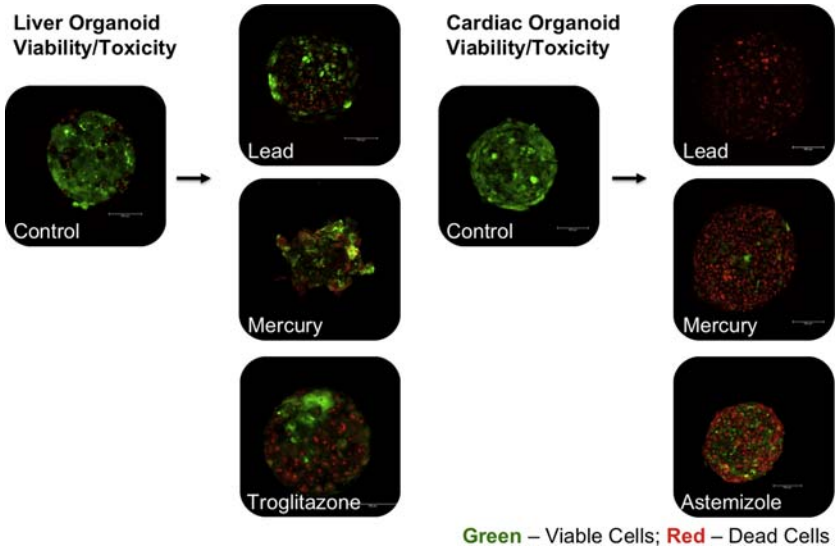

FIGURE 44.4 **Drug and toxicology screening in liver and cardiac organoids.** Live/dead stains show the presence of dead cells (*red*) after treatment with lead, mercury, troglitazone, or astemizole. Organoid diameter - 250 μm.

chemotherapeutics such as bleomycin, cause inflammation, toxicity, and irreversible fibrosis in the lungs or other organs. In most of these cases, toxicity and apoptosis lead to the release of proinflammatory cytokines such as tumor necrosis factor-α and interleukin-1 into the circulation. These molecules can cause a series of downstream responses, including recruitment of inflammatory cells, activation of fibroblasts, and changes in vascular protein expression and permeability (Fig. 44.4C). Integrated multiorganoid model systems, including integrated vasculatures, can model these complex, multiorgan drug responses in a reproducible and physiologically relevant manner, whereas single organoid systems cannot.

Cutting-Edge Body-on-a-Chip: The First Highly Functional Multiorganoid Systems

As described previously, there is a paucity of truly integrated multiorganoid platforms that are able to model and test the complex responses accurately to drugs, toxins, and disease across a range of human tissue types. However, progress is being made.

The Ex Vivo Console of Human Organoids Platform

Our team has developed an advanced, modular, multiorganoid integrated, body-on-a-chip system for use in drug development and toxicology screening. The multiorganoid body-on-a-chip platform is named Ex Vivo Console of Human Organoids (ECHO). This platform was initially developed to include four engineered tissue organoid types (liver, cardiac, vascular, and lung), which were developed independently and integrated into a single system that provides real-time monitoring of physiological responses to toxic agents and pharmaceuticals.

Using the ECHO platform, a comprehensive set of data was developed providing a characterization of each organoid. In general, the 3D organoids are bioprinted into platform microreactors using tissue type−specific supportive hydrogels. The result is an array of organoids suspended in a substrate containing tissue-specific, ECM-derived bioregulatory factors [36,91]. Liver organoids are fabricated using liver ECM−derived hydrogels that maintain viability and function in vitro for 4 weeks [91]. The production of important liver markers (e.g., albumin, multiple cytochrome P450 enzymes, epithelial cell−cell adhesion markers, dipeptidyl peptidase IV, organic solute transporter-α, etc.) have been confirmed and are stable over a month. These organoids respond to toxins such as APAP at appropriate doses and in a dose-dependent manner. The organoids may also be rescued by the clinical countermeasure for APAP intoxication, *N*-acetyl-L-cysteine (Fig. 44.4A). Cardiac organoids also remain for 4 weeks and beyond. These organoids demonstrate the transport of fluorescent dye molecules among cells within the organoids, indicating a high degree of cell−cell communication. The organoids beat spontaneously and change their beating rates appropriately in response to a variety of drugs. These kinetics are captured using an onboard camera system [92,93] and custom software for analysis (Fig. 44.4B). In addition, an engineered vasculature has been incorporated into the platform that responds to agents such as histamine by disrupting the endothelial cell monolayer

(Fig. 44.4C). This results in increased transendothelial transfer of larger−molecular weight molecules that are normally sequestered within the microfluidic circuit.

Several integrated, multiorganoid studies were performed using the ECHO platform. Experiments including integrated liver organoids, cardiac organoids, and endothelial modules in microfluidic devices (Fig. 44.5A and B) under common media demonstrated long-term viability and produced multiorganoid responses to drugs that largely mimic the responses that would be expected in an experimental animal, or even in a human. For example, Fig. 44.5C describes the effects of propranolol and epinephrine on cardiac organoids, both with and without integrated liver organoids in the microfluidic platform. Normally, epinephrine induces an increase in the beating rate in cardiomyocytes. Without liver, propranolol, a β-blocker, blocks the β1- and β2-adrenergic receptors, preventing an increase in the cardiac beat rate after epinephrine administration. However, in the integrated system that includes the liver organoids, propranolol is metabolized to an inactive form, resulting in the recovery of much of the epinephrine-induced increase in beat rate. To our knowledge, these experiments are the first interdependent multiorganoid studies performed successfully in a single integrated system.

The ECHO platform has been used to screen drugs that were withdrawn from the market owing to unanticipated toxicities. Because of the lack of accurate models to predict drug toxicity, many drugs have passed through preclinical studies and clinical trials, received FDA approval, and remained on the commercial market for years in some cases, before being recalled because they cause toxic effects in humans. Approximately 90% of drugs that were removed from the market were because of toxic effects in the liver and the heart. A panel of these drugs were tested in the ECHO platform. These include the drug troglitazone (Rezulin), an antidiabetic and antiinflammatory that was recalled for causing liver failure, and mibefradil, an ion channel blocker that was recalled for having fatal interactions with other drugs, including antibiotics. In our platform, troglitazone and mibefradil both result in liver toxicity. We also screened the drug rofecoxib (Vioxx), a nonsteroidal anti-inflammatory drug that was recalled because it caused serious vascular-based pathologies such as heart attack, stroke, skin reactions, and gastrointestinal bleeding. Astemizole, an antipsychotic that caused slowing of potassium channels, torsade de pointes, and QT prolongation. We also tested terodiline, a drug for bladder incontinence that caused QT prolongation and toxicity, in ECHO and showed functional changes and loss in viability among several cell types within the platform. The anticancer drug 5-FU and isoproterenol, a β-adrenergic agonist, both of which are known to induce cardiac toxicity, were evaluated in ECHO. Each of these drugs resulted in increased levels of cell death within the cardiac organoids in a dose-dependent manner. Using the onboard camera, beating effects were observed to decrease with dose increases as well. Effects on beat kinetics were detected at doses well below the toxic threshold. This is an important point, because drugs withdrawn from the market for cardiac toxicity are generally not withdrawn for killing cells in the heart, but rather for causing changes in heartbeat kinetics.

Other Body-on-a-Chip Programs

In addition to the ECHO platform initiative, a variety of other high-profile programs have been working to develop integrated systems. In particular, the Advanced Tissue-Engineered Human Ectypal Network Analyzer (ATHENA) program designed a millimeter-scale multiorganoid system, and a program sponsored by the Defense Advanced Research Projects Agency (DARPA) sponsored a 10-organoid project [94].

These projects have stressed aspects of microphysiological systems that are often different from the main goal of ECHO (i.e., organoid integration within a common microfluidic circuit). Specifically, the ATHENA program, which is based in Los Alamos National Laboratory, developed a system composed of four organs: liver, heart, lung, and kidney [95]. These organoids are three orders of magnitude larger than the ECHO organoids. This scale allows for more relevant mechanical testing and the system fluid volume is sufficient to collect samples that may be analyzed using standard clinical diagnostic equipment. The DARPA program, based in Harvard's Wyss Institute, is working with a collection of 10 organoids, including representations of endocrine, gastrointestinal, immune, musculoskeletal, and reproductive tissues [96].

In addition, the NIH are supporting a major organ-on-a-chip program through the National Center for Advancing Translational Science, the National Institute for Biomedical Imaging and Bioengineering, the National Cancer Institute, the Eunice Kennedy Shriver National Institute of Child Health and Human Development, the National Institute of Environmental Health Sciences, the NIH Common Fund, and the NIH Office of Research on Women's Health. However, the NIH initiative differs in that the funding is divided among a variety of individual research laboratories; between them, they are working on organoid representations of a wide range of tissue types [97]. The

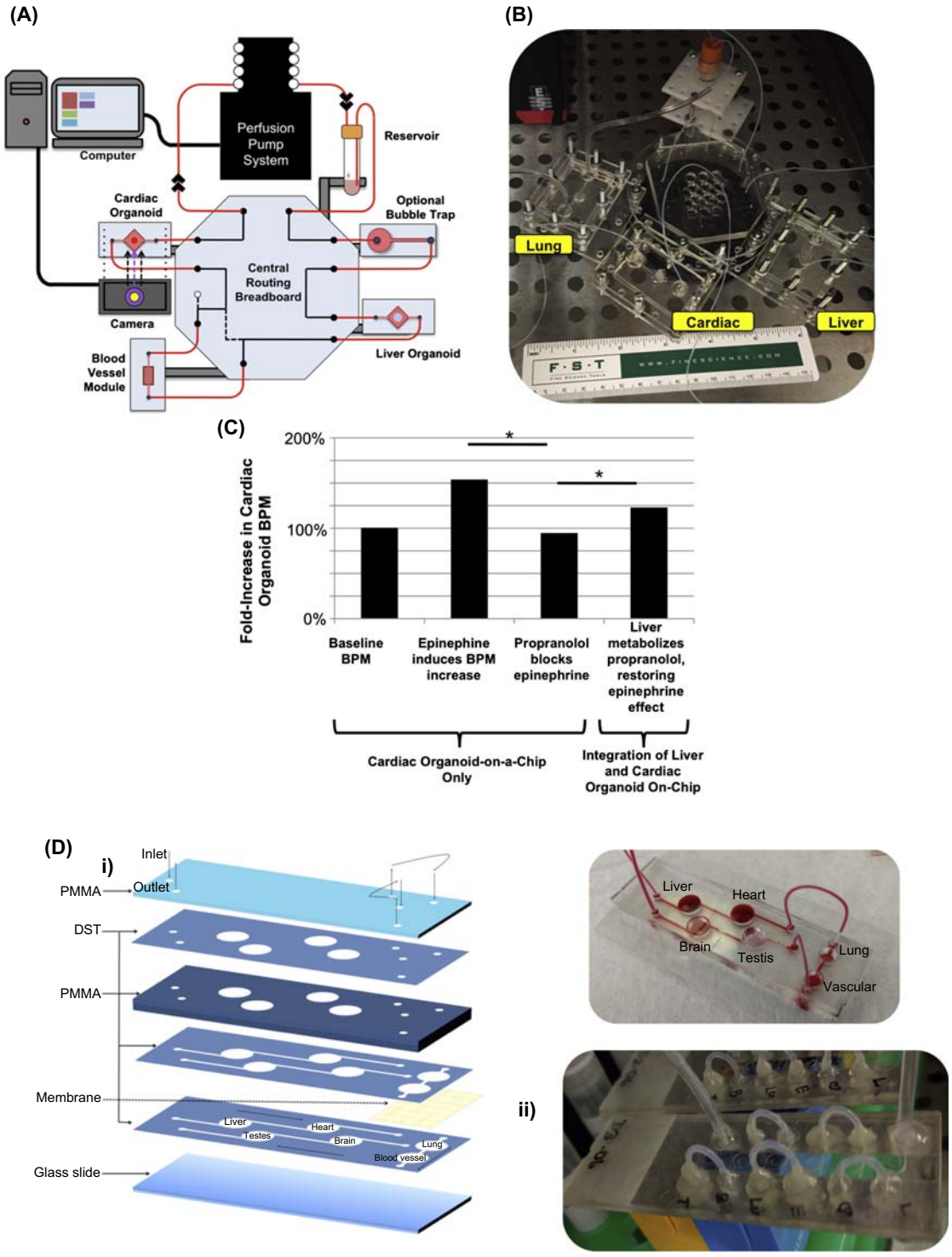

FIGURE 44.5 **Multiorganoid body-on-a-chip.** (A) Depiction of a liver, cardiac, and vascular organoid-containing body-on-a-chip platform. (B) Photograph of the three-organoid system. (C) Description of the effects of propranolol and epinephrine on cardiac organoids, with or without liver organoids, illustrating the importance of multiorganoid systems. Without liver, propranolol, a β-blocker, blocks cardiac beating increases by epinephrine. However, with both organoids present, propranolol is metabolized by the liver organoid, resulting in a measurable epinephrine-induced increase in beating rates. (D) (i, ii) Future body-on-a-chip platforms for increased capabilities for linking multiple organoids within a single circulatory system. *BPM*, beats per minute; *DST*, double-sided tape; PMMA, poly(methyl methacrylate).

program stresses the derivation of organoids from induced pluripotent stem cells (iPSCs), and many of the organoids developed by members of this program are excellent models for specific tissue types. However, the potential for integrating these organoids into a multiorganoid system remains to be seen.

Organ-on-a-Chip Systems for Personalized Precision Medicine

Numerous in vitro systems are being developed for general drug development screening, but few have been developed to benefit specific patients. This is an unmet clinical need, because finding the most effect drug and dose for a specific patient is often a trial-and-error process. With personalized organoid models (Fig. 44.6A and B), therapies can be screened using a patient's own cells in a 3D tissue organoid system. For example, accurately predicting a patient's tumor progression and response to therapy is one of the most challenging aspects of oncology. Prescribed treatments are often made based on the general success rate of a drug within a population, not on the specific response that may be expected within an individual. The concept of precision, or personalized, medicine has evolved to address these problems by using the patient's genetic profile to identify "druggable" targets for treatment [98–100]. However, in real-world practice, the results of this approach to personalized medicine do not reach the desired goals [101]. After identifying key mutations through genetic profiling, physicians can still be left with a variety of drug options, with no concrete data regarding potential side effects or actual drug effectiveness in the patient. As such, there is a clear need to develop tools that can predict the response of individual patients to drugs [102,103]. Our group is working to develop a multiorganoid platform that contains patient-specific tumor organoids, and in which drug therapies that are selected based on genetic profiling can be tested for efficacy. By using

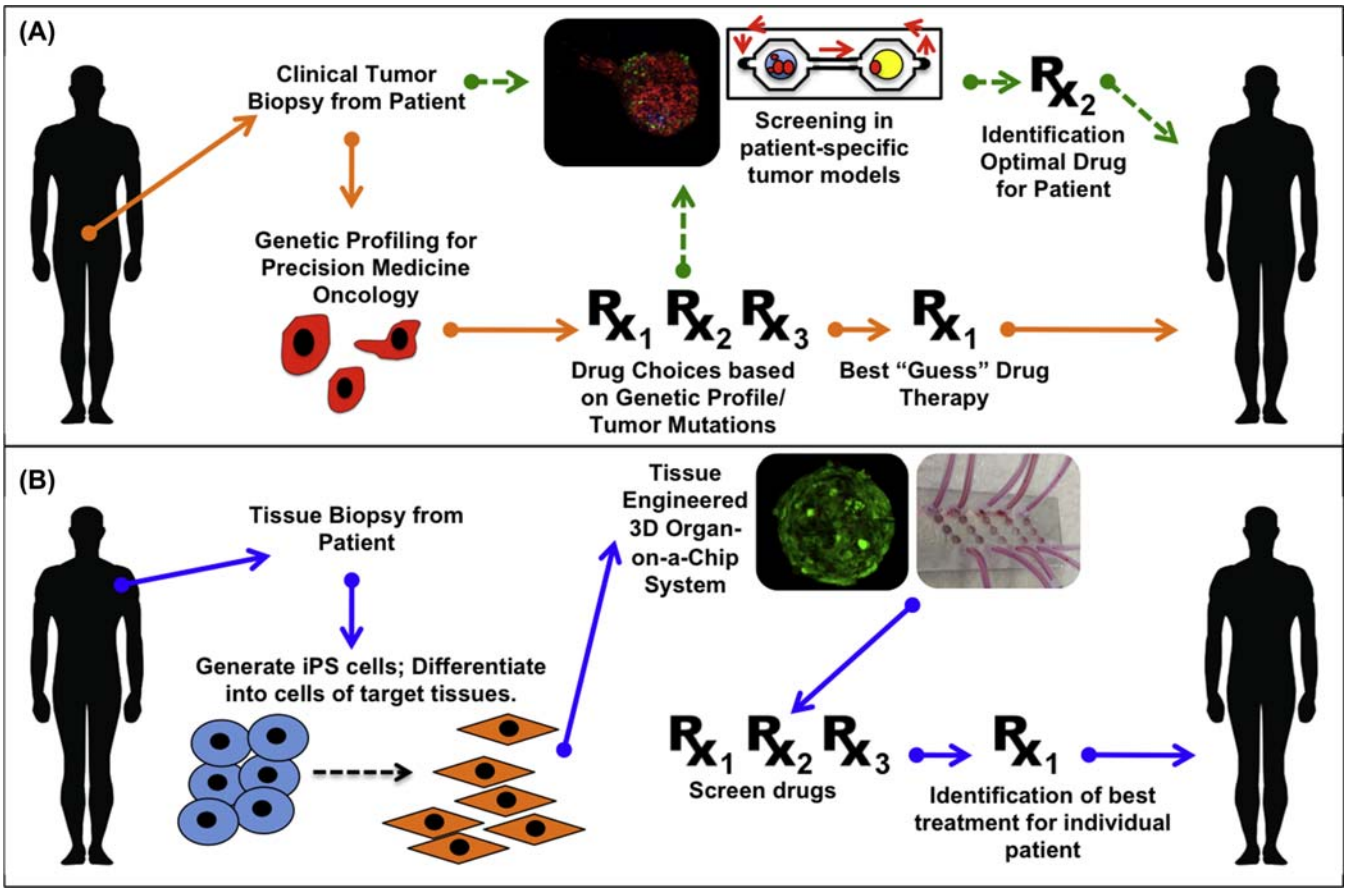

FIGURE 44.6 **Employing biofabricated tissues in personalized medicine.** (A) In personalized precision medicine for cancer patients (*red arrows*), a potential list of drugs is determined based on mutations found in the tumor genetic profile, from which best-guess therapies are prescribed. In the future, cells from tumor biopsies could be used to create in vitro tumor models specific to a given patient (*green arrows*). Potentially effective drug therapies can then be screened in the models, thus identifying the optimal drug therapy for that patient in terms of both safety and effectiveness. (B) In genetic diseases, cells can be harvested from alternative tissues, such as skin, translated into induced pluripotent stem cells (iPS), differentiated into cells of the tissue of interest (e.g., lung or heart), and bioengineered into three-dimensional (3D) organoids and organoid-on-a-chip systems, after which generic and genome-specific drug therapies can be screened for the original patient.

microfluidic devices, a circulatory system containing multiple tissue organoids may be fabricated. This may allow for the prediction of tissues to which metastatic cells will migrate [84]. By combining all of these capabilities described, it may soon be possible to generate accurate prognoses regarding tumor progression and metastases and to develop an informed treatment plan that is personalized to each patient's tumor. In the near future, iPSC technology may allow for the generation of an array of patient-specific normal tissue organoids that could provide information about the sensitivity of these tissues to a specific treatment regimen. This would enable the selection of an agent that reduces stress on the healthy tissues within a cancer patient. These exciting and powerful new in vitro technologies may soon revolutionize the treatment of cancer and other diseases.

CONCLUSIONS AND PERSPECTIVES

Although the benefits of multiorganoid systems are clear, several challenges remain regarding their acceptance and widespread deployment for drug development and personalized medicine. Most single cell−type systems do a respectable job in mimicking a few specific aspects of in vivo physiology and are amenable to high-throughput drug screening. The few single- and multiorganoid platforms, including multiple cell types within each organoid, provide a much better model for human physiology but have not yet been optimized for high throughput [104]. As such, these more complex systems are best-suited for evaluating drugs that are in the later stages of development. Many groups are actively developing strategies for multiorganoid systems and automating their production [14]. A significant reduction in size, automated fabrication, improved onboard biosensing, and in-line diagnostic technology would greatly increase the throughput potential for multiorganoid platforms across all potential applications [92,93,105,106]. Another perceived challenge in the development of advanced, multiorganoid, platforms is the requirement of a common cell medium to support a wide variety of cell phenotypes. Typically, human primary cells and iPSC-derived cells require complex, highly specialized media formulations that are tailored to each specific cell type. Surprisingly, there is growing evidence that 3D cell constructs are intrinsically supportive and much less reliant on complexed media supplements or serum. Our group has repeatedly demonstrated the maintenance of a variety of cell types in human cancer models [84,107] using serum-free medium and customized hydrogen substrates [36,91,108]. Even more remarkable is the ECHO platform described earlier in this chapter. In this platform, up to six organoid types, each containing up to five human primary cell types, have been maintained under a serum-free common medium for at least a month with minimal loss in viability and function. It is likely that the organoids themselves produce the autocrine and paracrine factors that are required for long-term viability and function. The compact architecture, scant interstitial space between cells, and accumulation of ECM proteins would allow these factors to become concentrated within each organoid and diminish reliance on exogenous factors delivered through the medium. Multiorganoid body-on-a-chip technology is advancing at a rapid pace and is likely be deployed soon for drug screening [10]. These platforms have significant utility in many areas and will dramatically change the way in which precision medicine, cancer modeling, and drug development are performed.

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CHAPTER

45

Bioreactors in Regenerative Medicine

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INTRODUCTION

The goal of regenerative medicine is to restore the structure and function of damaged, diseased, or lost tissues and organs. Most human tissues have limited regenerative capacity after injury and heal with scar formation or inflammation [1]. Accordingly, approaches to regenerative medicine use tissue-specific cells, biomaterials, and biologically active molecules to enhance regenerative responses and promote the formation of matured and functional tissues. In particular, tissue constructs and whole organs can be produced in vitro by providing physiologically relevant culture conditions and by subjecting the cells to biomimetic physical and biochemical cues [2]. By closely recapitulating the native environments of tissue development, bioreactors can provide conditions for growing fully functional and viable tissues and organs for transplantation in vitro (Fig. 45.1).

DESIGN CONSIDERATIONS FOR CREATING BIOREACTORS

When appropriate conditions are provided, cells can survive and grow, and eventually form tissue units outside the body. Conventional two-dimensional (2D) cell culture protocols enable the efficient generation of cells with controllable properties and phenotypes, but simple cell assemblies grown on 2D surfaces would be insufficient for applications in regenerative medicine, which typically require three-dimensional (3D) and functional tissue constructs. Using a bioreactor, physiologically functional 3D tissues can be bioengineered in vitro by recreating the microenvironmental and macroenvironmental niche conditions to cells seeded into scaffolds such as the extracellular matrix (ECM) [3]. To this end, several physical and biological aspects must be considered in designing bioreactor systems to produce tissues or even whole organs with desired physiological properties.

To promote cell viability, proliferation, and differentiation, it is essential to provide adequate amounts of oxygen, nutrients, and biochemical factors to the cells or tissues cultured in a bioreactor. In addition, metabolic waste generated by the cells must be removed effectively to further facilitate cell and tissue growth. As the cell density and tissue size increase, mass transfer through bioengineered constructs becomes challenging. Under static culture conditions, diffusion is the major mechanism for the transport of nutrients, most critically oxygen. However, diffusional transport is efficient only within a superficial layer (usually about 100–200 μm) of the 3D tissue constructs, which can be at least several millimeters to centimeters thick. Accordingly, to enhance the efficiency of gas and mass transfer, convective flow can be generated within bioreactors by stirring, rotating, or perfusing culture medium [4–6].

Mechanical forces can regulate the physiology of cells, in particular osteocytes, chondrocytes, cardiomyocytes, and skeletal muscle cells, via mechanotransduction pathways by which mechanical signals are converted into biochemical signals [7]. Thus, tissue formation and development can be promoted by mechanical stimuli, motivating the use of various mechanical stimulation regimes to regenerate tissues and organs with desired properties. For example, tissue-engineered cartilage with enhanced functionality was produced by applying physiological levels of compression to hydrogels seeded with articular chondrocytes [8,9]. Similarly, enhanced cell proliferation and tissue organization were achieved by repeatedly stretching scaffolds embedded with heart cells [10] or skeletal muscle cells [11]. Shear forces induced by fluid flow increased osteogenic expression and chondrogenic responses [12].

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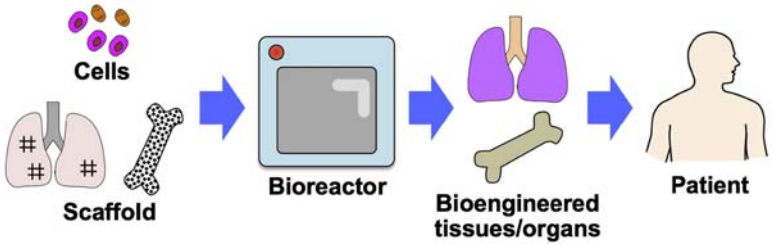

FIGURE 45.1 **Schematic of tissue or organ regeneration in vitro using bioreactor systems.**

Cell behaviors such as proliferation, differentiation, and migration can also be influenced by electrical stimulation in electrically excitable cells [13]. In particular, when appropriate electrical stimulation is applied, cardiomyocytes cultured in vitro, combined with biomaterials such as collagen, can differentiate and form into functional cardiac tissue constructs [14]. Development of contractile and electrophysiological function in bioengineered tissue constructs can be facilitated by cyclic electrical stimulation and mechanical strain. Tissue contractions resulting from the stimuli can promote cell elongation, orientation, organization of connexin-43, and the formation of structural features similar to those of native myocardium. The electrical stimulation also increases the expression of myosin heavy chain, creatine kinase-MM, and cardiac troponin-I in the bioengineered cardiac constructs. Notably, the positive effects of the electrical stimulation on tissue development strongly depend on the time of its initiation, which indicates that the timing of stimulation must be carefully determined to bioengineer cardiac tissues [15].

In addition to physical stimuli, regeneration of tissues and organs of interest requires the addition of biochemical cues that can modulate cellular and tissue behaviors [16], in particular, soluble biochemical factors such as growth factors and cytokines that are provided to local cells through secretion from neighboring cells or through supply from the blood instruct cells during development and tissue regeneration [17]. Because the concentration and spatial gradient of the biochemical factors strongly influence the microenvironment that dictate the fates of the cells, tight control of the biochemical parameters within the in vitro system is essential to obtain tissue and organs for specific applications.

In the body, cells reside in a complex 3D microenvironment that is composed of tissue- and organ-specific cell types, the ECM, and physical and biochemical factors Accordingly, the regeneration of tissue and organs for transplantation requires 3D scaffolds, in which cells seeded are provided with a biomimetic niche microenvironment that can promote the formation of functional tissue or reconstruct viable organs [18]. Various synthetic ECM materials such as poly(ethylene glycol) and poly(vinyl alcohol), which are typically soft and porous and have a high water content, have been employed as scaffolds that can facilitate the organization of cells into a 3D architecture by presenting the necessary structural, physical, and biochemical characteristics [19]. On the other hand, biological scaffold materials obtained from the native ECM have been shown to promote regeneration of functional tissues greatly [20]. Although the mechanisms mediating the cell behavior by the natural ECM are not well-understood, ECM derived from many different tissues such as heart, blood vessels, skin, and tendons have been tested in both preclinical and clinical applications [18].

Methods that allow monitoring of the tissue growth and maturity, and generation of tissue- and organ-specific functionalities are needed for the successful applications of bioengineered tissues and organs in regenerative medicine [21]. Typical assays that are used to analyze the structure and biochemical composition of the tissue, such as histology and microscopy, involve tissue-sampling procedures, which is destructive in nature. On the other hand, advanced imaging techniques such as molecular imaging and deep-tissue imaging techniques allow noninvasive assessment in vitro and enable real-time and long-term evaluation of bioengineered tissues and organs.

LUNG BIOREACTORS

Bioengineering Functional Lungs

For many patients with end-stage lung disease, lung transplant is the only definitive treatment option available. Although timely organ transplant is important to increase the life span and overall life quality of the recipients, this treatment procedure is significantly hampered by the severe shortage of viable donor lungs [22]. Furthermore, less than 20% of donated lungs are acceptable for transplant whereas the rest are unsuitable because of poor lung

conditions caused by various reasons including contusion or edema [23]. Accordingly, to increase the number of lungs suitable for transplant, tissue-engineering methods are being developed to salvage donor lungs that are marginally unacceptable and rejected for transplant. In these approaches, endogenous cells in the donor lungs are replaced with fresh cells to restore the lung function to a level acceptable for transplant.

Important processes for lung regeneration using tissue-engineering technologies include (1) removal of the cellular components from the donor lungs (i.e., decellularization), and (2) repopulation of the resulting lung scaffold with the new cells such as lung airways cells or stem/progenitor cells (i.e., recellularization) [24]. The 3D lung scaffold provides organ-specific environment with structural and biochemical cues for the transplanted cells to facilitate lung remodeling and regeneration. In particular, functional ECM components preserved in the decellularized lung matrix, such as collagens, elastin, fibronectin, laminin, and glycosaminoglycans, can promote cell engraftment and differentiation during recellularization.

For the accelerated regeneration of viable and functional lungs, a bioreactor is an essential component that can offer suitable environments to the bioengineered lung [25]. For instance, normothermic (37°C) perfusion and sterile support of the lungs during reconditioning can be achieved by using a bioreactor. An ideal bioreactor would provide mechanical and biochemical cues to the lungs mimicking physiological stimuli during the native lung development. Furthermore, physically and biologically relevant conditions including the air−liquid interface and breathing movement should be generated in the lung bioreactor to promote precisely regulated differentiation and organization of the transplanted cells in the lung scaffolds created by decellularization.

Bioreactors for Regeneration of Small Animal Lungs

Because of their relatively easy access, lungs harvested from small animals such as rodents [26,27] have been widely used to establish protocols for lung tissue engineering (Fig. 45.2A). Accordingly, various lung bioreactors

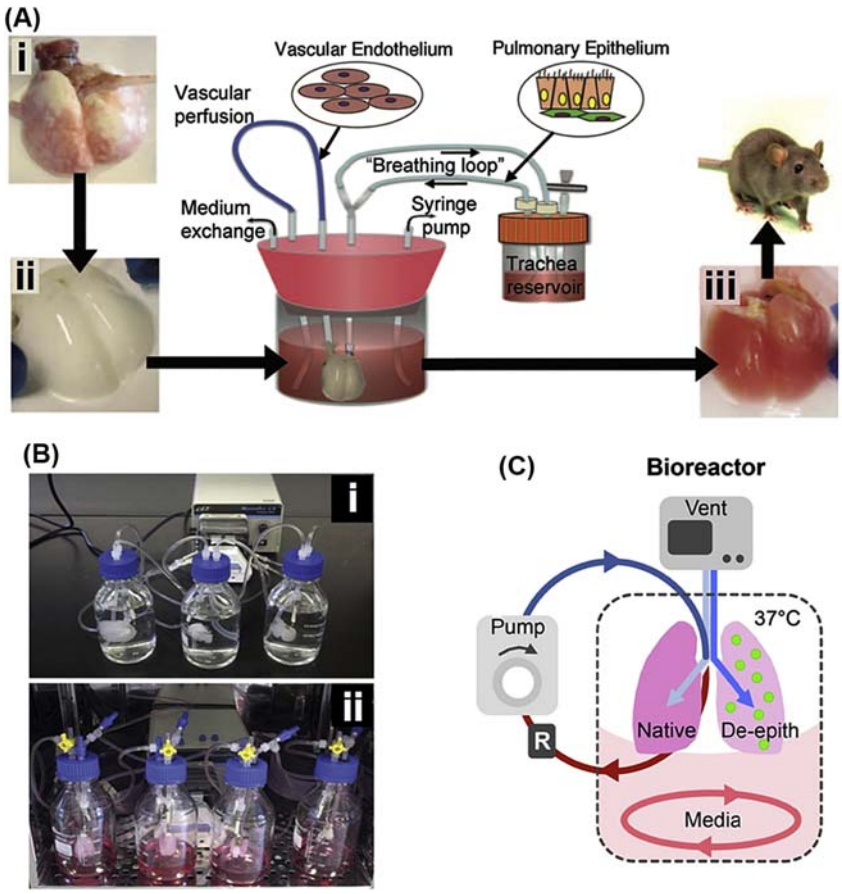

FIGURE 45.2 **Bioreactors for ex vivo regeneration of small-animal lungs.** (A) Schematic for lung regeneration process [26]. (B) Photograph of bioreactors for (i) decellularization and (ii) recellularization of rat lungs [28]. (C) Schematic of a bioreactor for selective cell replacement in the rat lungs [29]. *De-epith*, de-epithelialization.

designed for small animals have been evaluated for the long-term preservation of explanted lungs during cell-based organ regeneration via decellularization followed by recellularization (Fig. 45.2B). It is imperative that the lung bioreactors provide physiologically relevant conditions (e.g., sterile conditions, isothermic perfusion, and air ventilation) to the lungs throughout the regeneration procedures.

While lungs are kept in a bioreactor, chemical or biological decellularization agents can be perfused sequentially through the airways and vasculatures to remove entire cellular components from the lungs [30]. This perfusion-based decellularization requires precisely controlled fluid flow to maximize preservation of the structural integrity and biological activity of the lung ECM. Decellularization agents include detergents such as 3-([3-cholamidopropyl] dimethylammonio)-1-propanesulfonate, sodium dodecyl sulfate, and Triton X-100 and enzymes such as nuclease, trypsin, and collagenase. After cell removal, the lungs are seeded with therapeutic cells (e.g., epithelial and endothelial) through the airway and vasculature. During recellularization, the lung is ventilated and perfused, respectively, via ventilator and pump integrated into the bioreactor, recapitulating physiological lung developmental conditions (Fig. 45.2C).

A novel bioreactor for the rat lungs was developed in which one lobe of the lung could be bioengineered while the other one remained intact to serve as a control [29]. This bioreactor allowed removal of the epithelial cell layer (de-epithelialization) from the airways of the small animal lungs (i.e., rat) while the underneath lung tissue was preserved (Fig. 45.3D). The selective cell removal was achieved by introducing a mild decellularization solution through the airway while the solution entering the blood vessels was quickly cleared by continuously perfusing the vasculature. As a result, the vasculature network in the lung could be maintained undisrupted, allowing structural and biological integrity of the blood−gas barrier that facilitated bioengineering of functional lungs after reepithelialization.

Bioreactors for Regeneration of Large Animal Lungs

To generate bioengineered lungs on a clinically relevant scale, bioreactors have been developed that could maintain the function of large-animal lungs (e.g., porcine and human) during ex vivo regeneration [31,33,34] (Fig. 45.3A and B). Similar to bioreactors for small animal lungs, these clinical-scale bioreactors allow sterile, normothermic perfusion and ventilation to promote lung regeneration and functional recovery. In addition, large-lung bioreactors have been further optimized to support the lungs for a sufficient time to allow diagnostic and therapeutic interventions that would be needed to improve marginal lungs. Automated lung perfusion and ventilation with integrated sensors (e.g., flow, pressure sensors) and lung monitoring components (e.g., video cameras) into the bioreactor would be desirable to achieve more tightly controlled lung regeneration.

Whereas ex vivo lung perfusion (EVLP) has typically been used to preserve donor lungs during transport, its utility for assessment and reconditioning of marginally unacceptable donor lungs has also been pursued (Fig. 45.3C). Benefits of using EVLP include the recruitment of collapsed lung areas, removal of bronchial secretions, and clearing of clots from the circulation. In addition, because the lungs are connected to the EVLP system externally, direct and targeted treatments of the lungs are possible via endotracheal and intravascular routes. Owing to the absence of other organs in the circuit, higher doses of therapeutics for accelerated disease treatment can be administered to the lungs supported by EVLP (Fig. 45.3C). However, the lack of other organs also implies that the metabolic clearance and systemic factors are absent. Nevertheless, EVLP systems have served as excellent platforms for human lung support and bioengineering [35]. The adaptation of EVLP for reconditioning marginal donor lungs increased the total number lung transplants at least by 15% [36].

In Vivo Bioreactors for Lung Regeneration

Allogeneic solid organ transplants typically require nonspecific and lifelong immunosuppressive therapy to prevent and treat organ rejection. Although immunosuppression improves graft survival, the patient becomes susceptible to infections, in particular to pulmonary infections that may cause morbidity and mortality [37]. An effective approach to reducing the immunogenic response would be using the recipient as an "in vivo bioreactor," in which donated organs or tissues are implanted heterotopically, thus taking full advantage of the natural regenerative capacity of the recipient's body [38]. This elegant method was demonstrated for trachea (Fig. 45.4A), with the donor trachea implanted subcutaneously into the recipient's forearm for over 9 months. During this time, the neovascularization occurred and the endogenous mucosal lining was partially replaced with the recipient's own buccal mucosa. After transplantation of the regenerated trachea, the immunosuppressive therapy was suspended and the recipient's

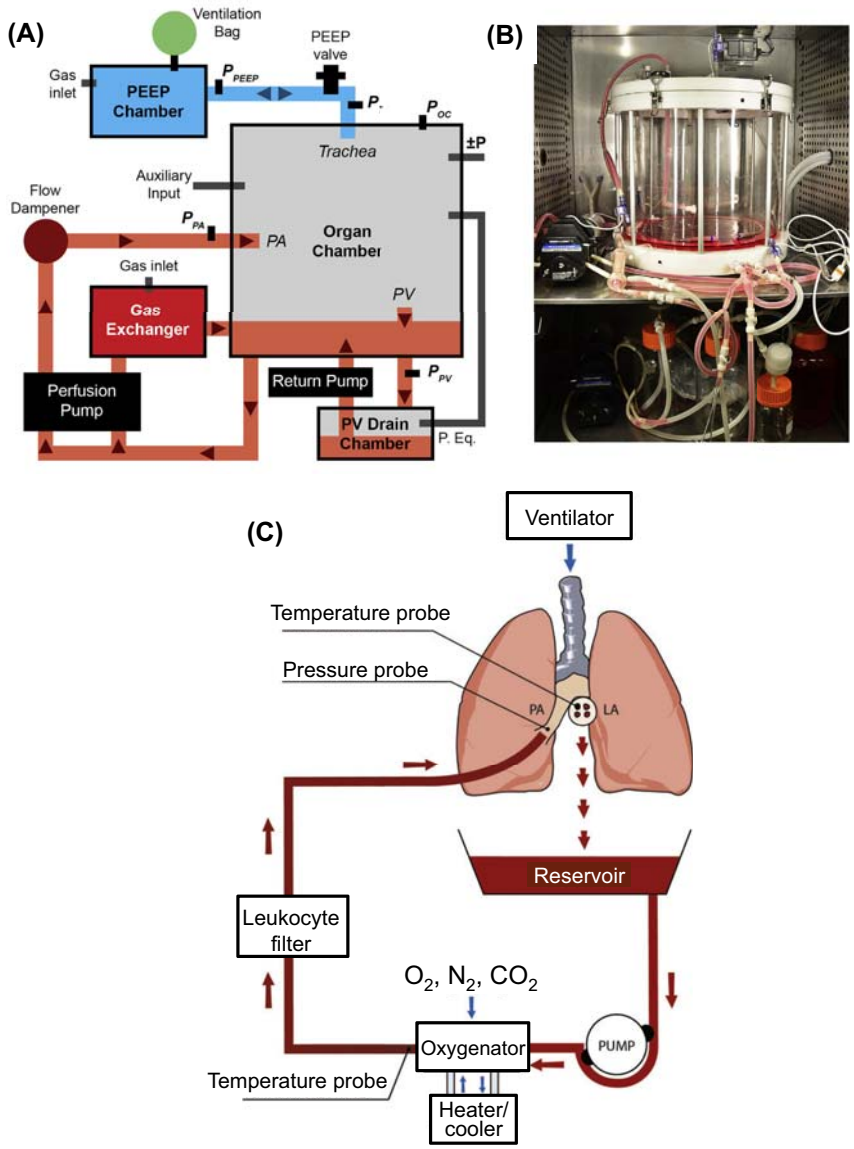

FIGURE 45.3 **Bioreactors for ex vivo regeneration of large-animal lungs.** (A) Schematic layout of a clinical-scale lung bioreactor and (B) photograph of the bioreactor setup in an incubator [31]. (C) Schematic drawing of an ex vivo lung perfusion (EVLP) unit [32]. *LA*, left atrium; *P.Eq.*, pressure equilibrium; *PA*, pulmonary artery; *PEEP*, positive end-expiratory pressure; *PV*, pulmonary vein.

buccal mucosa gradually grew over the graft. Significantly, the allogeneic trachea was stable and functional for several years even after immunosuppression was discontinued [39,41].

Smaller tissues or organs such as trachea and bone segments can be heterotopically regenerated within the recipient's own body before orthotopic transplantation. However, this in vivo approach can be difficult for the regeneration of solid organs such as whole lungs or liver owing to their large size and the need for perfusion. A potential solution for this can be cross-circulation (extracorporeal circulation), in which donor organs are connected externally to the recipient's circulatory system during organ recovery or repair [42,43]. Using a preclinical swine model, this novel cross-circulation approach has been demonstrated for prolonged lung support during therapeutic interventions undertaken to recover the injured donor lungs [40] (Fig. 45.4B and C). Because the donor organ is placed outside the recipient's body, the organ is more accessible for diagnostic and therapeutic interventions that can improve the

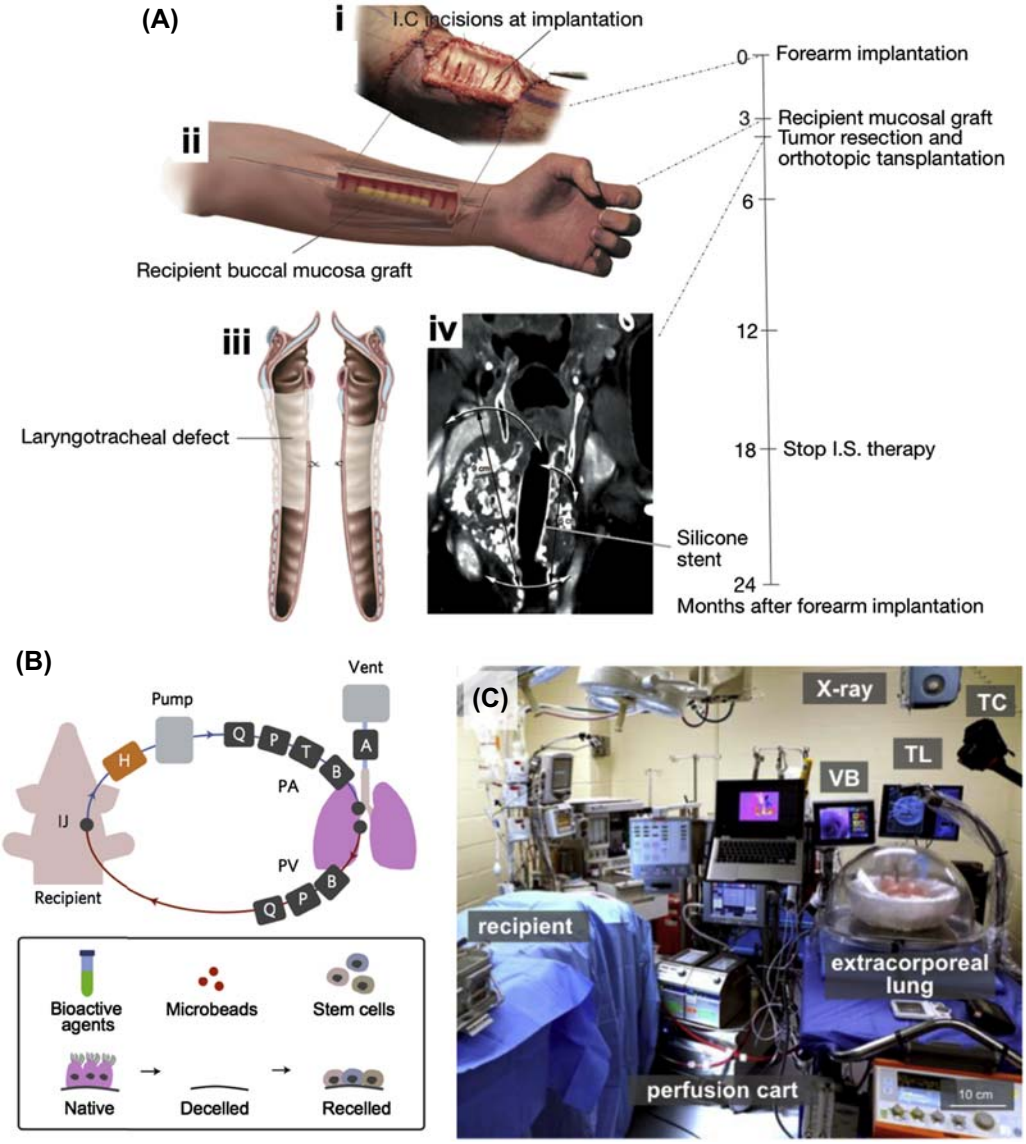

FIGURE 45.4 **In vivo bioreactors** (A) Tracheal allotransplant after revascularization in a heterotopic position (forearm) of the patient [39]. (B) Cross-circulation circuit for regeneration of donor lungs ex vivo supported by the recipient and (C) operative setup for the cross-circulation [40]. *H*, warm water jacket; *I.C*, incision; *I.S.*, immunosuppression; *IJ*, internal jugular vein; *PA*, pulmonary artery; *PV*, pulmonary vein; *TC*, thermal camera; *TL*, time-lapse camera; *VB*, video bronchoscopy.

organ conditions, and minimally invasive evaluation of the lung function recovery. In addition, lung regeneration through cross-circulation can be accelerated when combined with the cell-replacement therapy using the patient's own cells, because the recipient provides the necessary biochemical cues needed by the transplanted cells.

Bioreactors for Study of Lung Biology

Lung regeneration and repair involve complex processes for which the underlying mechanisms are not fully understood [44]. More detailed study of lung development can enable the establishment of effective treatment modalities for a number of respiratory complications including immature lungs in preterm babies and inflammation-induced disruption of alveologenesis. In addition, such study would also inform strategies for stem cell–based ex vivo lung regeneration [45]. Microengineered lung models in microfluidic culture devices (i.e., "lung-on-a-chip") have great value for high-throughput in vitro studies of the lung at the molecular, cellular, and tissue levels [46–48] (Fig. 45.5A). Thus, these biomimetic lung-on-a-chips can recapitulate the structure, function, and microenvironment of human lung, permitting the study of lung development, regeneration, disease, and responses to drugs.

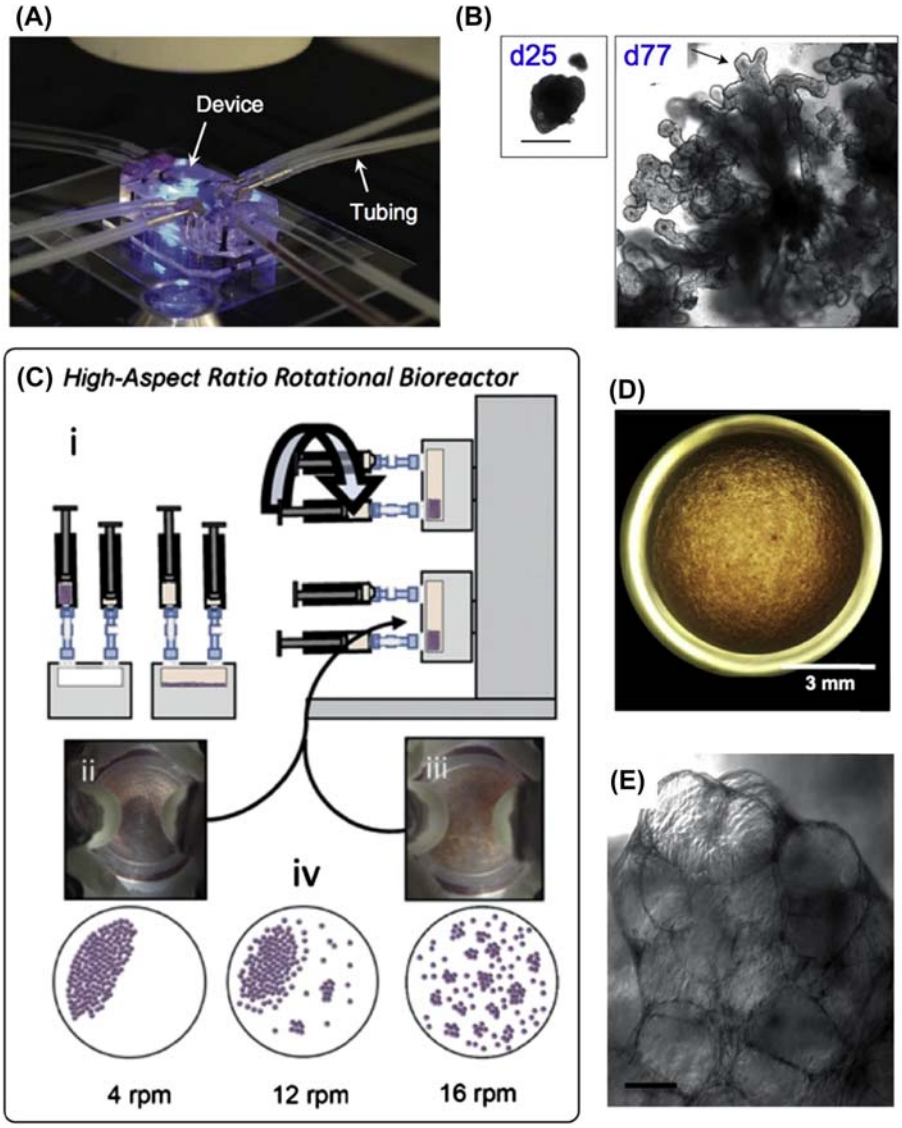

FIGURE 45.5 **In vitro (iv) devices for studies of lung development and regeneration.** (A) A lung-on-a-chip model for microengineering small airways [48]. (B) Lung bud organoids grown in vitro in a hydrogel [49]. (C) Bioreactor for lung organoid generation [50]. (D, E) Bright-field microscopy of a lung organoid [51].

When provided with suitable biochemical and physical conditions, stem cells can be formed into 3D tissue structures (i.e., organoids) that contain self-organized cell clusters. For example, human pluripotent stem cells cultured in 3D hydrogel (e.g., Matrigel) formed lung bud organoids resembling branching human airways and early alveolar structures [49] (Fig. 45.5B). Whereas model systems such as 2D monolayer cell cultures, 3D cell spheroids, and tissue explants have limitations in recapitulating the complex and multilevel human physiology, organoids can faithfully recapitulate many biological parameters, providing a unique opportunity to perform more physiologically relevant studies in vitro. Using custom-built bioreactor systems, generation of organoids can be facilitated in terms of organoid number and size, establishing the use of organoids in applications of drug testing, regenerative medicine, and disease modeling [51] (Fig. 45.5C–E).

Evaluation of Bioengineered Lungs

Various imaging and tissue processing techniques are used to evaluate the bioengineered lungs and assess their structural and biochemical integrity [21]. For example, removal and repopulation of cells such as epithelium of the

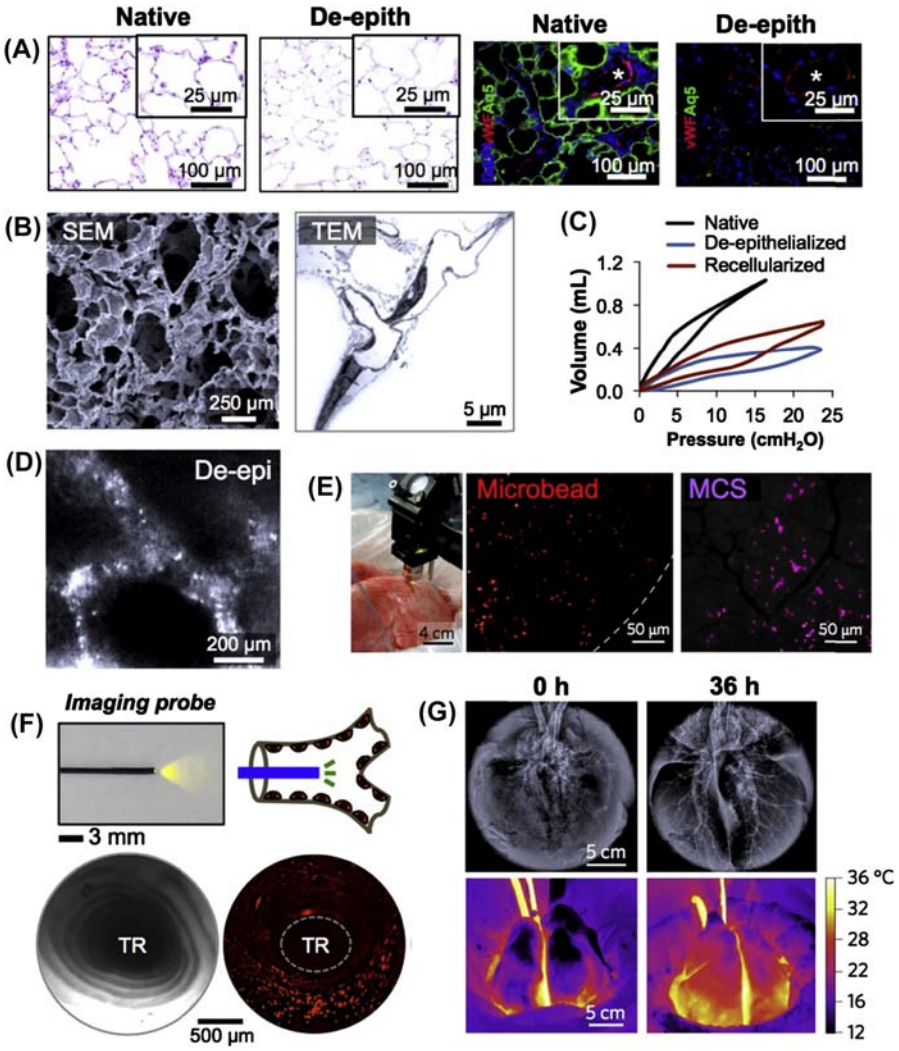

FIGURE 45.6 **Evaluation of the microstructure and composition of the lung tissue.** (A) Hematoxylin–eosin and immunofluorescence staining of lung tissue samples of airways. (B) Scanning electron microscopy (SEM) of native and de-epithelialized (De-epith, De epi) lungs and transmission electron microscopy (TEM) of alveoli. (C) Pressure–volume measures of lungs. (D) Microscopic imaging of fluorescent microbeads (diameter = ~1 μm) in the lung capillaries [29]. (E) Transpleural imaging of labeled microbeads or mesenchymal stem cells (MSCs) in the lung parenchyma [40]. (F) Imaging of MSCs seeded onto the rat trachea [52]. (G) X-ray and thermal imaging of the porcine lung during regeneration [40]. *TR*, trachea.

lung can be visually assessed by hematoxylin and eosin staining of the tissue samples obtained from the lung. In addition, immunofluorescence staining can reveal the presence or absence of biological molecules in the lung tissue (Fig. 45.6A). Detail about cell and tissue structures can be probed by using powerful imaging techniques such as scanning electron microscopy and transmission electron microscopy, which provide high-resolution images [29] (Fig. 45.6B).

To evaluate the lungs without the need for tissue sampling, nondestructive and real-time assessment have been employed [29,40,52]. A simple method to determine the quality of the bioengineered lung is to measure the pressure–volume relation of the lung, which can provide lung compliance and resistance (Fig. 45.6C). In addition, the structural integrity of the blood vessels can be evaluated by directly monitoring traveling paths of microbeads (diameter of approximately 1 μm) added into the blood flow (Fig. 45.6D). Similarly, cells added into the lung parenchyma (Fig. 45.6E) or airways (Fig. 45.6F) can be visually inspected using a custom-built microscope and optical-fiber imaging probe, respectively. Airway recruitment and blood perfusion of the lung during regeneration can be inspected using X-ray and thermal imaging (Fig. 45.6G).

BONE BIOREACTORS

Bioengineering Bone

Bone tissue has an inherent ability to regenerate, except in critical-sized defects caused by congenital abnormalities, trauma, or disease. Autologous grafts are the reference standard treatment, but these grafts are limited by the tissue volumes that can be harvested and patient morbidity. Engineered bone substitutes rely on bioreactors to provide nutrients throughout the large tissue constructs and to deliver the mechanical stimuli needed to promote bone development [53]. In this section, we examine bioreactor design for bone tissue engineering, including an advanced example of clinical translation, and the important role of bioreactors in delivering dynamic loads to cells within the engineered bone. Like lung, bone is a vascularized organ, and we summarize efforts to use the body as its own bioreactor to mitigate the challenge of vascularizing implants. Finally, we consider the use of bioreactors as platforms for biological studies of bone development and disease, and how nondestructive modalities may be used to monitor bone structure and function within bioreactor environments.

Nonperfused Bioreactors for Bone Regeneration

Static tissue culture relies on diffusion to deliver nutrients and biophysical stimuli to tissue, resulting in cell viability and matrix deposition only at the border of a 3D construct. To drive nutrient distribution in bone tissue engineering, two types of bioreactors for dynamic culture have been introduced: rotating wall vessels that use concentric, rotation cylinders to create laminar flow; and spinner flasks that use a magnetic stirrer or similar device to create flow through constructs suspended in the flask. Both setups have shown improvements in cell viability and distribution compared with static culture; however, in both cases, flow remains restricted to the periphery and necessary nutrients and signaling cannot reach deep within critical-sized scaffolds [54].

Perfusion Bioreactors for Bone Regeneration

Perfusion bioreactors, in which media are pumped throughout the construct, have shown improved cell distribution and viability and matrix production throughout large 3D scaffolds [55,56]. Perfusion bioreactors pump media from a reservoir throughout a construct within a liquid-tight vessel. Flow may be directed around the outside of a construct, or it may be forced directly through a construct by press-fitting the scaffold within the bioreactor casing. The latter setup offers superior mass transport, although flow patterns need to be customized to fit the construct geometry [57]. Perfusion bioreactors have been used for engineering bone from various stem cell sources, including embryonic [58], induced pluripotent [59], bone marrow–derived [60], and adipose-derived stem cells [61,62].

Bone marrow–derived and adipose-derived mesenchymal stem cells (MSCs) have shown particular promise for clinical translation, because both have demonstrated success in perfusion-bioreactor engineering of large-animal, anatomical bone replacements [60,61] (Fig. 45.7). These custom-shaped bioreactors relied on preoperative imaging of the targeted defect from which a mold was fabricated. Two elastomer pieces were then molded and press-fit onto a decellularized bone scaffold, which was milled to the anatomical shape and loaded with stem cells (Fig. 45.7A). To ensure optimal mass transport and shear stress throughout the construct, computational flow simulations were used to determine the channel size and placement (Fig. 45.7B). The engineered bone closely resembled the resected, native bone after 3 weeks in culture (Fig. 45.7C) and guided generation of new bone over 6 months in vivo in adult pigs (Fig. 45.7D). In contrast, soft issue formed in the condylectomy control (Fig. 45.7E). Custom perfusion bioreactors may be extended to implement multiple chambers for engineering adjacent tissues, such as engineering the articular cartilage surface on bone to form whole joints [63,64].

An important advantage of perfusion bioreactors is the ability to control the media flow throughout engineered bone, not only for mass transport but also to introduce appropriate shear stresses to cells for bone development. Previous reviews reported on the mechanobiology of bone development and the use of bioreactors to recapitulate physiological shear stresses [53,65]. Shear stresses are critical to bone formation and resorption via mechanotransduction in osteocytes, which communicate via paracrine signaling with osteoblasts and osteocytes to regulate matrix turnover [66]. Shear stress was also shown to direct osteogenic differentiation of MSCs [67]. Several studies attempted to recapitulate the physiological stresses seen by cells in native bone using perfusion bioreactors [56].

However, because no method exists for measuring the locally applied shear stress directly at the cellular level in 3D, studies rely on computational simulation of flow rates to determine local stresses. An important study established predictive correlates between the perfusion rate and the local osteogenic behavior of human MSCs in

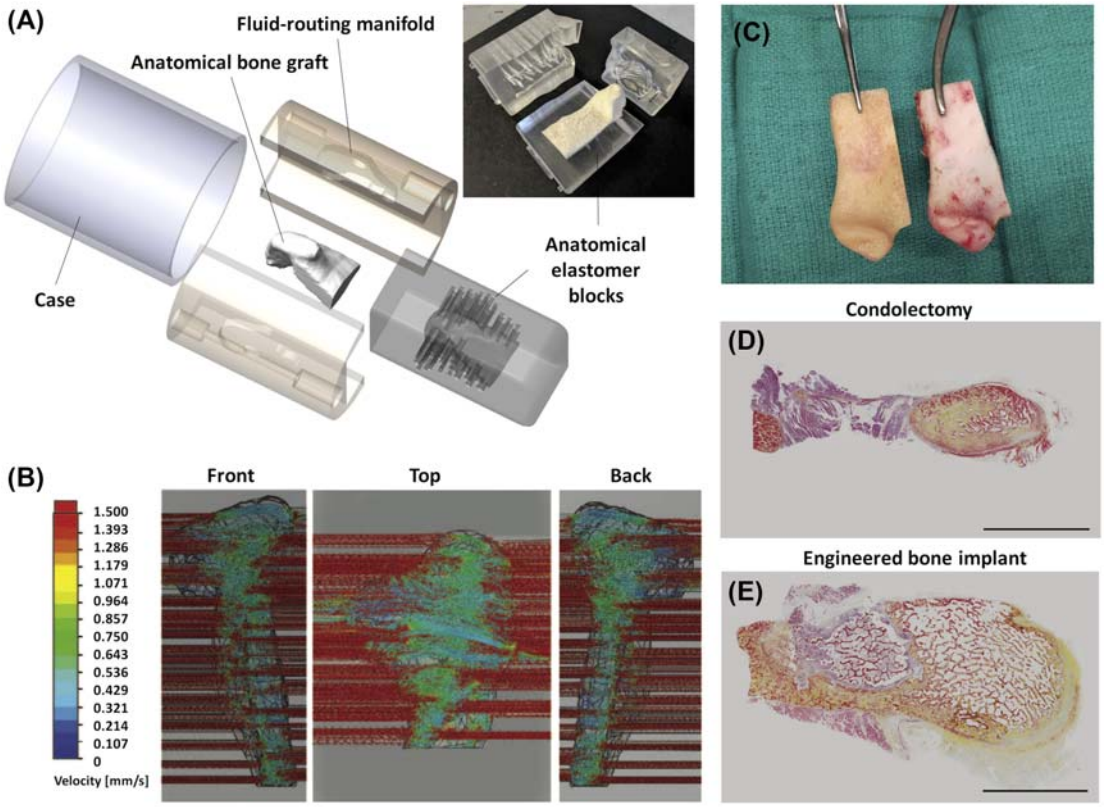

FIGURE 45.7 **Engineering anatomical bone grafts using a perfusion bioreactor.** (A) The bioreactor consists of two polydimethylsiloxane - blocks with incorporated channels, two manifolds, and a casing, providing controlled perfusion through the scaffold. (B) Channel diameters and spacing were designed by flow simulation software to provide a desired interstitial flow velocity for a given shape and size of graft. Simulations revealed a uniform flow velocity throughout the volume. (C) Comparison of resected native bone and engineered graft shows closely matched geometry. (D) Movat pentachrome stain of explants from pigs undergoing condylectomy showed fibrous tissue filling the defect; (E) engineered scaffolds guided development of new calcified tissue in the defect [61].

decellularized bone matrix [57]. Computational simulations yielded the independent contributions of oxygen delivery and shear stress to the resulting osteogenic behavior, including gene expression, matrix deposition, and cell proliferation. A range of flow rates from 400 to 1800 μm/s resulted in stresses of 2−20 mPa, respectively; although this is more than an order of magnitude lower than stress values reported in vivo [53], stresses were expected to increase as more matrix was produced and pore size correspondingly decreased.

Although many perfusion bioreactors introduce steady, continuous flow to engineered bone, there may be advantages to mimicking the oscillatory or pulsatile loading experienced by bone tissue in vivo [55]. External loads are applied to cells via a combination of matrix strain and interstitial fluid flow through lacunar-canalicular spaces [68]. A study examined the direct effects of oscillatory flow on MSCs using a parallel-plate bioreactor setup, in which the pressure is spatially constant throughout the setup, as confirmed by computational fluid dynamics (Fig. 45.8A). By testing various load magnitudes and frequencies, a regime of 2 Pa at 2 Hz was identified to have the greatest upregulation of osteogenic expression in MSCs [69], similar to the loads experienced by human cells in vivo during jogging.

Beyond fluid flow, external intermittent compression may be applied for mechanosignaling for bone development. A compression bioreactor setup, including an array of platens driven by a linear actuator and interfaced with a 24-well plate, was used to introduce periodic strain cycles and investigate mechanical regulation in a bone tumor model [70] (Fig. 45.8B). Computational simulation of the local pressure and flow velocity throughout the porous decellularized bone matrix (modeled as a poroelastic material) allowed the correlation of local pressure and matrix strain with gene expression. A new paradigm for dynamic loading in a bone bioreactor was reported in which nanoscale vibrations were introduced to MSCs via a magnetic plate [71] (Fig. 45.8C). Simulations of the displacement within the constructs revealed a constant force magnitude across the construct surface, and MSCs undergoing nanovibrations generated mineralized matrix despite the absence of physiological matrix stiffness

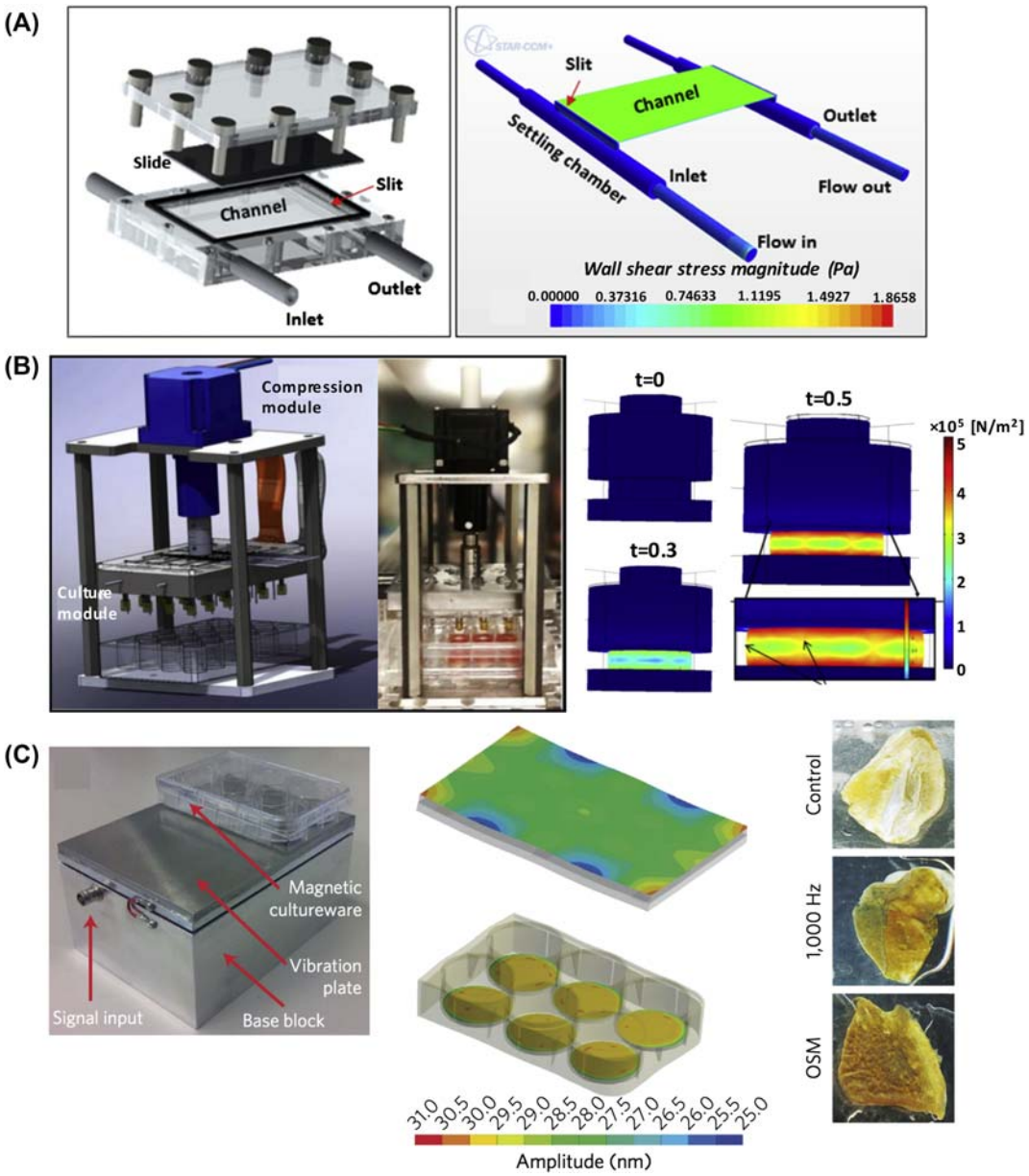

FIGURE 45.8 **Bone bioreactors with advanced loading regimes and corresponding mechanics simulations.** (A) Oscillatory flow bioreactor (*left*) and stress field simulations (*right*) [69]. (B) Compression bioreactor (*left*) and simulated stress field (*right*) [70]. (C) Vibrational bioreactor, simulated nanoscale displacements of the magnetic plate (*top*) and six-well plate (*bottom*) used for culture, and resulting constructs without stimulation, with mechanical stimulation, and with osteospecific media (OSM) [71].

(soft collagen gel served as a matrix material). These diverse approaches to loading bone tissue in bioreactors showed that mechanical effects on bone development must be considered in designing bioreactors.

In Vivo Bone Bioreactors for Solving the Vascularization Problem

One of the greatest challenges in producing viable engineered bone substitutes is the generation and anastomosis of a functioning vascular network. Like lung, bone is a highly vascularized tissue and efforts to incorporate vasculature during in vitro development have fallen short of promoting vascular integration upon implantation. A potential approach to overcome this is to use the body itself as a vascularized bioreactor and develop the bone in an ectopic location before orthotopic implantation [72–74]. This "in vivo bioreactor" principle has the additional advantage that the body provides a further supply of stem cells and growth factors to the construct, avoiding overmanipulating

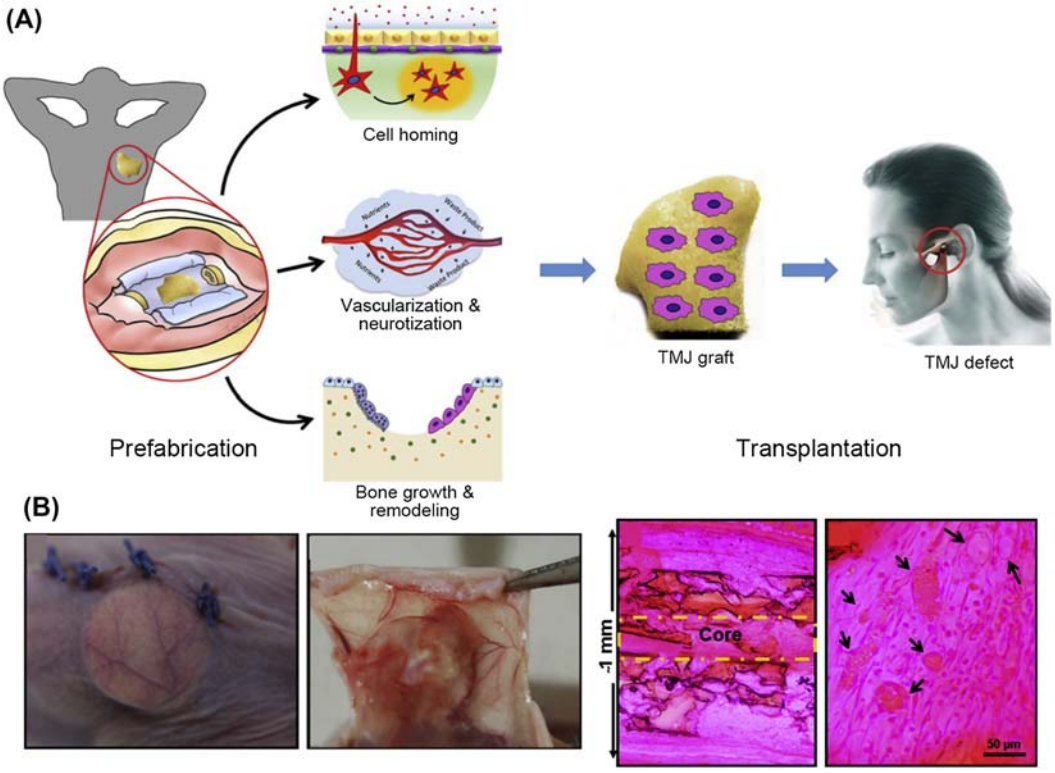

FIGURE 45.9 **In vivo bone bioreactor.** (A) The in vivo bioreactor paradigm: ectopic implantation prepares a construct for successful orthotopic implantation [73]. (B) Layered nanofibrous–ceramic constructs implanted subcutaneously in nude mice to promote vascularization (left). Hematoxylin–eosin histology shows that vascularization occurred all the way through the core of the scaffold [75]. *TMJ*, temporomandibular joint.

the tissue ahead of implantation, which could simplify the clinical translation of engineered bone replacements [73] (Fig. 45.9A). Since it was originally introduced, in vivo bioreactor studies have taken advantage of the highly vascularized environment in the periosteum, the connective tissue layer surrounding the bone [69]. The successful formation of mineralized, compact bone was demonstrated by 6 weeks after implantation, in rabbit periosteum. Given the relatively invasive nature of this implantation, subcutaneous implantation has also been explored for its vascularization potential. To promote vascular ingrowth, a layered construct was engineered using MSCs within electrospun polycaprolactone nanofibers, an angiogenic layer composed of collagen–fibronectin gel with endothelial cells, and an osteoconductive layer containing hydroxyapatite particles. After 4 weeks of subcutaneous implantation in nude mice, vasculature was present throughout the core of the scaffold [75] (Fig. 45.9B).

Bioreactors for Studying Bone Development and Disease

Bioreactors for bone were originally introduced to improve mass transport to cells when culturing in large 3D constructs. However, the opportunity to introduce a controlled mechanical and biological environment to realistic 3D bone constructs has allowed sophisticated models of bone development and disease within bioreactors. For instance, advanced mechanobiology studies may be carried out in perfusion bioreactors. A study examined the synergistic effects of matrix stiffness and shear stress on the osteogenic differentiation of progenitor cells. Decellularized bone matrices were coated with various ratios of collagen–hydroxyapatite to vary matrix stiffness and were loaded with MSCs [76]. The authors modeled flow through the constructs to estimate shear stresses and found that oscillatory flow is needed to promote cell viability and osteogenic behavior in long-term culture, whereas matrix stiffness had to be optimized separately for the greatest osteogenic potential. The controlled environment provided by bioreactors could also be used to study fracture repair under different biological regimes. For instance, static culture of hypertrophic chondrocytes improved long bone repair via endochondral ossification in the rat model, in contrast to osteoblast-laden scaffolds cultured in a perfusion bioreactor that promoted intramembranous ossification [77].

Bioreactors have also shown advantages in modeling the bone tumor microenvironment to understand tumorigenesis and drug efficacy better [70,78]. A study that used a dynamic compression bioreactor showed that mechanical loading affects the tumor's drug sensitivity in an engineered model of Ewing sarcoma [79]. Effects of shear stresses on tumor cell homing to bone during metastasis have also been studied. Spinner flask culture upregulated interleukin-24, which was shown to decrease the viability of prostate cells in engineered bone compared with static coculture [80]. The role of compression in inhibiting tumor-mediated osteolysis and metastatic tumor formation in bone by breast cancer cells was observed in a compression bioreactor for bone engineering [81].

Bioreactors also enable the modeling of joint disease for drug screening applications [82]. By scaling down and simplifying the bioreactor design, the osteochondral interface has been studied "on a chip" using microfluidics to introduce appropriate media to the two tissues [83]. A similar dual-compartment design was implemented to grow osteochondral tissues from human MSCs, and interleukin-1β induced tissue degeneration [84]. This platform is suitable for testing disease-modifying osteoarthritis drugs.

Monitoring the Environment and Tissue Development Within Bioreactors

The abilities to sense and visualize the inner workings of bioreactors are crucial for determining their faithfulness in recapitulating the physiologic environment and for evaluating proper tissue development. In the context of bone bioreactors, several structural, mechanical, and biological assessment tools have been proposed to monitor tissues and cells nondestructively.

Microcomputed tomography (μCT) is a nondestructive imaging modality that visualizes tissue structure in 3D. Because of the high absorption of X-rays by mineralized tissue, it may be used to quantify formation of bone longitudinally and has been a primary modality for this application [85]. Some studies have performed μCT imaging directly within perfusion bioreactors [86,87] without the need to remove the construct from its media circuit and sterile environment. Such longitudinal imaging can provide feedback to 3D tissue formation during culture, enabling the adaptation of biophysical stimuli as needed. Direct μCT monitoring within a perfusion bioreactor also allowed the relation of flow rate to the formation of mineralized tissue by human MSCs in a decellularized bone scaffold [88]. Longitudinal μCT has been used to investigate the effects of the degree of matrix curvature on new bone formation in 3D silk fibroin constructs [86] (Fig. 45.10A). Scaffolds with channels of varying degrees of curvature, to mimic the matrix turnover in vivo, were cultivated with cells in perfusion and static bioreactors to study fracture healing.

Optical imaging, including bioluminescent and fluorescent imaging, offers molecular specificity and may be performed nondestructively to investigate cell behavior within bone bioreactors. Bioluminescence has been used to track stem cell behavior in bone defects in vivo in mice (Fig. 45.10B). MSCs were transfected with two variants of luciferase, one used as a constitutive promoter to quantify cell number and another as a cell differentiation reporter for osteoblast lineage [89]. This approach is a promising avenue to understand stem cell fate better in engineered bone. Noninvasive, optical assessment of oxygenation in a bioreactor has also been achieved by measuring the oxygen-dependent phosphorescence lifetime of microprobes within the media of a perfusion bioreactor [91].

The authors found a decrease in oxygen within tissue constructs under static culture conditions, in contrast to stable oxygen levels in the perfusion setup. However, the technique provides no spatially resolved information; it offers a single oxygen measurement at each time point. Another disadvantage of optical modalities is a limited depth penetration, which restricts imaging to thick, opaque constructs.

Owing to both shear stresses and matrix strains, mechanical effects on bone development have received increased attention, but many studies lack a method to visualize the effects of mechanics directly within dynamic bioreactor environments. A bioreactor system was demonstrated that is capable of real-time mechanical conditioning (dynamic, uniaxial strain, and electrical stimulation) of nanofibrous constructs and the simultaneous monitoring of local strains [90]. Fluorescent beads and MSCs were electrospun directly into fibers, and fluorescence microscopy along with digital image correlation techniques were used to map local strains (Fig. 45.10C). Such a system may provide new insights for bone mechanobiology and allow tracking of the mechanical development of bone tissue in future studies. Elastography, which uses medical imaging including ultrasound, magnetic resonance imaging (MRI), and optical coherence tomography, may also have a role in mechanically conditioned engineered tissues [92].

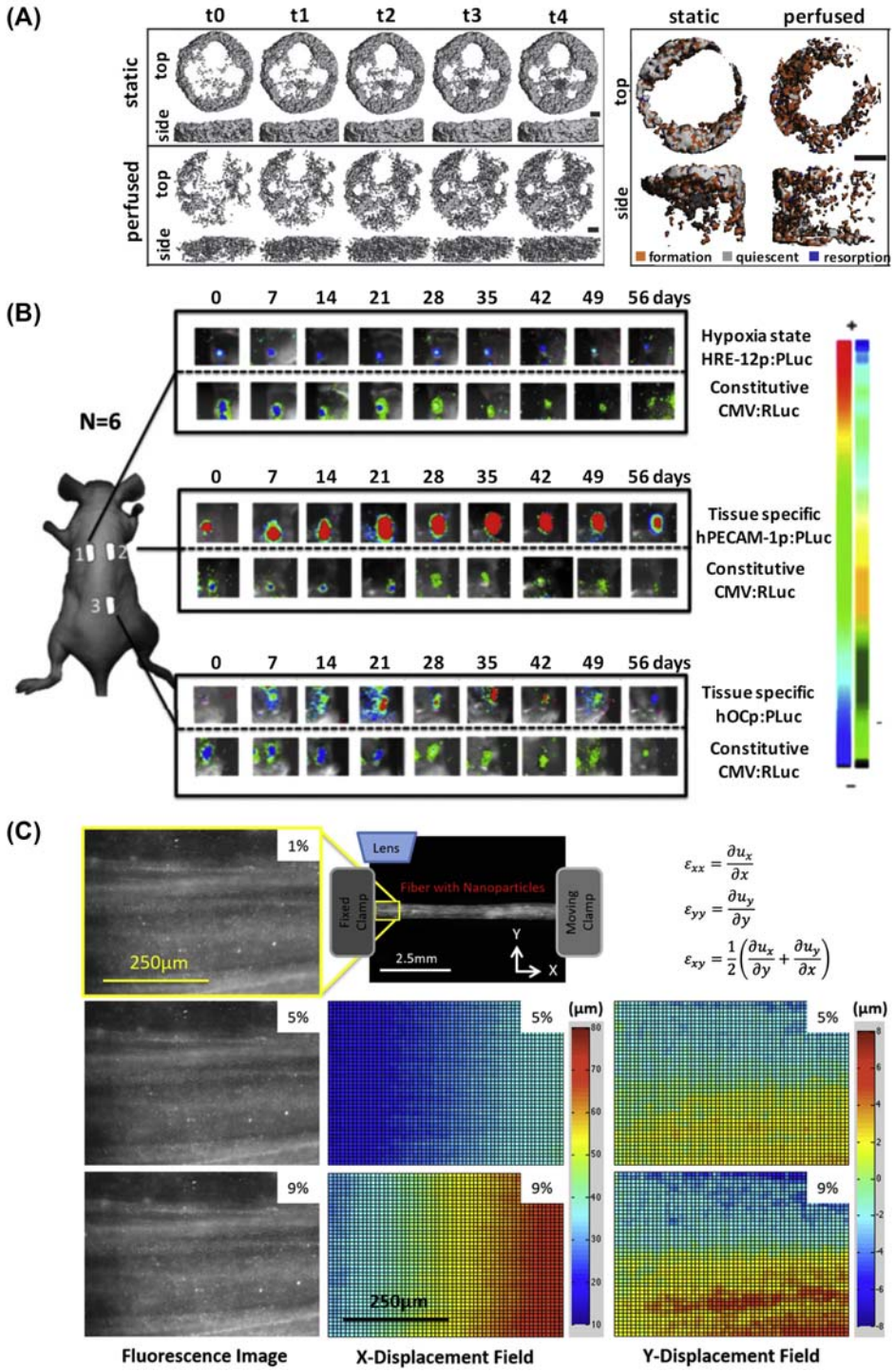

FIGURE 45.10 **Image-based monitoring of tissue and cell behavior in bone models.** (A) microcomputed tomography monitoring of bone deposition in silk fibroin scaffolds with and without perfusion. Color-coded overlays of different time points show areas of new growth and resorption [86]. (B) Bioluminescent imaging of human mesenchymal stem cell (MSC) differentiation in demineralized bone scaffolds implanted in a mouse model. MSCs were transduced with reporters for cell number (renilla luciferase [RLuc]) and/or cell differentiation (photinus luciferase [PLuc]) toward osteoblast lineage (OC), endothelial lineage (human platelet endothelial cell adhesion molecule [hPECAM]), or hypoxic expression (HRE) [89]. (C) Optical monitoring of local strain in nanofibrous constructs in a mechanical bioreactor. Fluorescent beads were incorporated into the fibers to track local displacements [90].

CHALLENGES AND FUTURE DIRECTIONS

Promising advances have been made in designing bioreactors that deliver essential nutrients and environmental cues to drive tissue development. However, several areas require improvement to enable the clinical translation of bioreactor technologies for regenerative medicine. As discussed here for lung and bone tissues, vascularization is critical for graft survival upon implantation. Using ectopic locations in the body as an in vivo bioreactor can mitigate some of the issues, but it may not always be practical. Other bio-inspired strategies should be considered that could be carried out in vitro to prime tissues for vascularization and contribute to success upon implantation. For example, harnessing the inflammatory response to promote angiogenesis has been explored in bone tissue constructs. Biomaterial-directed switching of the macrophage phenotype enabled upregulation of angiogenic factors and improved vascularization in a mouse model. Many bioreactor studies do not account for the inflammatory milieu, but it may be necessary to incorporate these cell types for optimal tissue development in the future.

In this chapter, we have introduced techniques that have been used to monitor the bioreactor environment and tissue development, but much work is needed in this space to interface bioreactors further with advanced imaging and sensing modalities. Although several modalities have been used to monitor cell fate and tissue development in vitro in monolayer culture [93], relatively few have been extended to monitoring the 3D complex environment within a bioreactor. Part of the challenge is the trade-off between the resolution needed to capture cellular-scale events with the field of view needed to monitor large, 3D constructs. MRI-compatible [94] and computed tomography−compatible bioreactors have been developed to monitor media flow and tissue structure, but they cannot track individual cells and have not been widely adopted for laboratory use, likely owing to the cost and size of the systems. Optical techniques including bioluminescence, fluorescence, multiphoton techniques, and optical coherence tomography may have an increasing role in real-time monitoring in future studies [93].

Real-time and nondestructive assessment of tissue and organ regeneration will be essential to the eventual automation of bioreactor control. Imaging and sensing readouts may be used in a feedback loop to signal inputs of environment cues (e.g., mechanical actuation, oxygenation) or delivery of biological factors. Toward this end, a computer-controlled perfusion bioreactor with integrated sensors for oxygen and pH was used to grow bone successfully for implantation in mice [95]. Eventual clinical translation will also require standardization and quality control; here, predictive computational models and automation are expected to contribute. Computational analyses and machine learning techniques may enable further the optimization of culture regimes, resulting in improved reproducibility in engineered construct quality.

Acknowledgments

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CHAPTER

46

Bioinks for Three-Dimensional Printing in Regenerative Medicine

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INTRODUCTION

The advent of three-dimensional printing (3DP) is revolutionizing approaches to fabricating tissue mimics for therapeutic replacement, drug discovery, and fundamental biological understanding. The potential niche for 3DP in tissue engineering is seemingly infinite, because we have at hand the ability to provide on-demand, fabricated, patient-specific designs rapidly and at a low cost. However, 3DP technology was first intended for industrial settings. The translation to tissue engineering applications is hindered by major hurdles that include technical printing issues and, more important, biocompatibility. Consequently, we are limited by the number of materials available that can satisfy both the 3DP and compatibility requirements. Here we provide a historical perspective of 3DP and summarize the different techniques, consider the important characteristic properties a bioink must have to fit bioprinting criteria, summarize bioink and biomaterial advancement used for 3D bioprinting, and discuss future directions to address current limitations for clinical impact.

Generally, printable biomaterials, or bioinks, are materials that can be used in 3DP techniques that include or will include biological features. The term "bioink" may lead to confusion because some may consider the material a bioink only if it is cell-laden or contains some matrix or matrix-mimicking component. However, we would like to expand its definition to encompass any printable material that (1) will interface with biological components (e.g., tissues, cells, proteins, growth factors) during or after the actual print, or (2) is involved in the structural construction of scaffolds that will interface with biological components.

Bioinks must comply with the 3DP technique as well as provide a biocompatible environment mimicking a desired tissue and ideally degrade controllably with no harmful by-products. Harmful by-products may not originate exclusively in degradation; they may also come from temporary bioinks that have structural roles during the printing process. Unfortunately, to satisfy these criteria, material properties work against each other and require some compromise between desired printability and satisfactory biological features. However, bioengineers have a selection of materials compatible with several different 3DP platforms. Therefore, in our discussion we will cover bioinks in the context of which printing technique is capable of printing with the described material. We will describe the diversity of available bioinks and biomaterials for 3DP under three main categories: (1) matrix or matrix-mimicking, (2) sacrificial, and (3) support [1].

FUNDAMENTALS OF THREE-DIMENSIONAL PRINTING

3DP is an additive manufacturing technique originally applied to plastic and metal manufacturing. It has progressed to adapt to biomedical engineering applications within the past few decades. Over 3 decades ago, Charles

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Hull patented a new technology for rapid prototyping, called stereolithography. This described system to fabricate three-dimensional (3D) constructs uses liquid photopolymerization to build desired objects in a stepwise manner and is considered the birth of 3D bioprinting [2,3]. 3DP and bioprinting have revolutionized the ability to create objects with any shape or size on demand. The exciting promise for medicine is that we can customize patient-specific tissue scaffolds, fabricate on-demand medical devices, and reliably reproduce constructs for high-throughput screening.

The key term that defines modern bioprinting is control. Casting approaches have surface resolution defined by the mold itself and no control over the internal structure of the casted sample. Similar salt leaching or electrospinning protocols for porous structures have little to no control over the internal pore size and distribution [4]. Modern technologies allow for control deposition and building; there is control over detailed characteristics such as the location and content of material deposition. This includes microstructures to bear stress, cell encapsulation, and growth factor or other biochemical functionalization. Being able to design the bulk shape and internal microstructure fully has allowed structures to be produced that more closely resemble nature's complexity: controlled internal microstructures (pores, gradients, and layering) [5–10], microchannel and micropatterning for prevascularization [11,12], and the simultaneous deposition of different types of materials [12–14] or different cell lines [6–8,12,15]. The technology allows for control and the mechanics are there to exploit; however, the hurdle to overcome is to define the bioinks that can be adequately used with these systems. An ever-expanding list exists of 3D bioprinting technologies (and bioinks to match) that are differently suited for specific desired materials or applications. These techniques vary based not only according to the materials (e.g., affected by cross-linking mechanisms to determine biocompatibility and/or mechanical properties) but to the resolution of the architecture and the speed of fabrication. Four 3DP methods have been adapted for biomedical application: (1) extrusion-based printing, (2) particle fusion-based printing, (3) inkjet printing, and (4) stereolithography or photopolymerization methods (Fig. 46.1). Technological advances have resulted in novel methods or modifications of these four, including acoustic droplet ejection, direct-write assembly, laser-guided direct writing, and 3D powder printing [3]. Each of these approaches has advantages and limitations. However, given an identified desired application, often one or a combination of technologies proves to match the sought biomaterial and architecture. A 2016 review by the Kaplan Lab [3] summarized the evolution of bioprinting and additive manufacturing technologies.

Extrusion-Based Printing

Extrusion-based printing is the most commonly employed method for 3DP, but it requires thermoplastic materials that are cell compatible only if they are printed at physiological temperatures. The technology forces a viscous ink

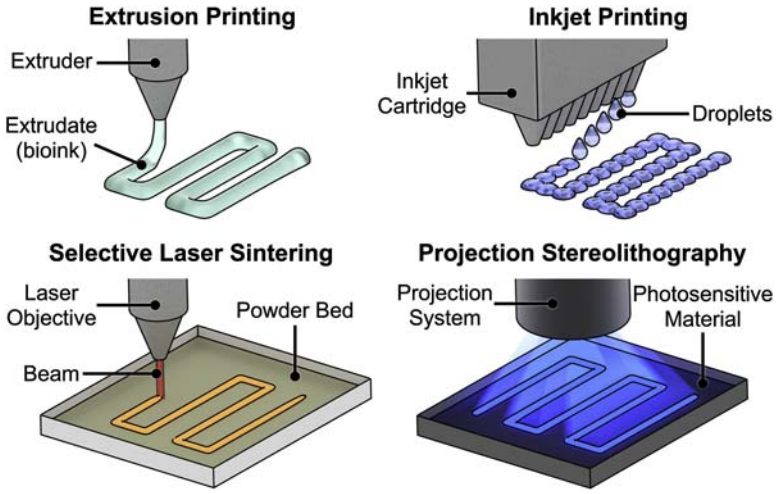

FIGURE 46.1 **Three-dimensional (3D) bioprinting techniques:** Extrusion-based printing deposits continuous filaments on a surface to build constructs layer by layer. Inkjet printing adapts inkjet cartridges to contain bioinks so that liquid droplets are formed on a surface that can quickly solidify. Selective laser sintering uses a laser to melt powder particles together to sinter a 3D object within the powder bed. Finally, projection stereolithography uses photosensitive liquid material that can be cross-linked with light exposure with controllable photomasks. *Reprinted with permission from Miller JS, Burdick JA. Editorial: special issue on 3D printing of biomaterials. ACS Biomater Sci Eng 2016;2:1658–61. Copyright 2017 American Chemical Society.*

through a nozzle that can solidify once deposited onto a build platform. The extruded material is deposited to form individual lines in a predefined path as dictated by a generated computer model to form a 3D object in a layer-by-layer fashion. Typically, each different ink is extruded out of the nozzle at a specific temperature and pressure so that the material can flow through the nozzle (e.g., polycaprolactone PCL] will extrude at 80°C and a pneumatic pressure of 400 kPa, whereas alginate can be deposited at 20°C [7]). Materials that are most commonly used for this technique possess a sharp solid-to-melt transition such that the material can flow and rapidly solidify once passed through the nozzle. Progress has been made in developing thermoplastics that extrude at lower, more physiological temperatures and pressures by exploiting shear thinning properties (i.e., PCL) [17]. Other approaches include using materials that can be extruded at lower temperatures or do not rely on thermal setting but require additional cross-linking mechanisms such as ionic bonding [18], pH alteration [19], or UV photopolymerization, among others. The inclusion of cells, however, can ultimately affect several aspects of the resulting print. Not only do materials need to be able to flow through the nozzle at low temperature, parameters such as high extrusion forces and narrow nozzle diameters, which usually improve the print's resolution for synthetics at high temperatures, affect cell viability. Decreasing the pressure and/or increasing the nozzle diameter may improve cell viability by reducing the cells' experienced shear stress but at the cost of potential nozzle clogging and print resolution. In addition, this technique has difficulty printing overhanging structures without supporting filler structures. The printed filaments can sag or even collapse without underlying support. Although some groups used extrusion printing with PCL or poly(propylene fumarate) (PPF) to create highly controlled porous scaffolds [20–22], others generated structures as a sacrificial template with similar extrusion-based methods to pattern architectures precisely that can be better-suited for some of the biocompatibility issues with this 3DP technique [11,23]. Overall, extrusion-based printing has been applied to many tissue engineering applications and is most widely available.

Selective Laser Sintering

Another printing technique, particle fusion printing methods such as selective laser sintering (SLS), uses a directed laser to raise the temperature of a powder material beyond its melting temperature to fuse or sinter the particles locally. The laser fuses each patterned layer in a repeated process to create 3D structures [24]. The construct's resolution is limited only by the laser resolution and the powder size. This technique has had its most successful applications to bone tissue engineering by sintering composite materials. Examples include ceramics, PCL, and hydroxyapatite [25–28]. Although the tissue engineering field has seen promise with this technique, the printed objects are difficult to incorporate with living cells and instead use a two-step process of first building the volume followed by adding cells. Moreover, there is difficulty in controlling porosity, which can affect the longevity of the biomedical application [3]. However, a major benefit is the technique's capacity to be self-supportive of complex printed structures, as the powder bed enables the printing of overhanging geometries. In addition, similar to extrusion-based printing approaches, groups use the technique of SLS additively [28] and sacrificially [29].

Inkjet Bioprinting

Inkjet bioprinting is considered the cheapest bioprinting technique. It was patented as one of the first strategies for cell printing [30,31]. Originally, inkjet bioprinters were created by replacing traditional 2D ink-based printers' cartridges with biological solutions. Instead of paper used as the recipient of the ink, a moveable stage added a third dimension to build a construct layer by layer [32,33]. Inkjet bioprinters can deposit picoliter droplets with positional accuracy less than 30 μm, allowing high-precision control in positioning different materials and cells into specific microenvironments [34]. To deposit liquid onto a substrate, some inkjet printers electrically heat the print head to a range between 200°C and 300°C. The high heat raises concerns regarding the viability and function of the cells after printing. Still, some studies using this technique show viability for mammalian cells and attribute survival to the short duration of the high-temperature exposure [35,36]. In inkjet printing, a liquid droplet is solidified after deposition onto a substrate, a process that must occur quickly to control the spatial resolution of the printed volume. Important material properties of the ink are the viscosity and the surface tension to determine the final shape, size, resolution, and accuracy of the print. Therefore, an important mechanism to consider to improve the final print is the cross-linkability of the bioink. Effective methods for this application are chemical, pH, or UV cross-linking [37,38]. There is a delicate balance of cross-linking the deposited droplets for rapid structural organization and to limit toxicity introduced to the cells. The chemical modification to achieve the desired cross-linking capability can decrease cell viability and affect the chemical and mechanical properties of the material. These changes can alter

the biomimicry that was relevant before modification [39]. In addition, inkjet printing uses a nozzle head to deposit the bioinks and therefore is limited by the possibility of clogging. Bioinks for inkjet printing should have low viscosities (below 10 cP) because the necessary pressures or heat that would eject higher viscosities would negatively affect cell viability [40].

Stereolithography

The last 3D printing technique to be discussed, stereolithography, brings us to the origins of 3D bioprinting. Stereolithography-based printing uses light as a tool to solidify liquid materials in a photochemical reaction. With a laser often used as the light source, a projection of light is shown onto the liquid material in a specific pattern to solidify the exposed region [41,42]. Using a light source, photosensitive material, and a controlled axis stage, one can print complex 3D structures. Because stereolithography depends on photosensitive material to print constructs, a limited number of biomaterials can be used. Common materials for this application are poly(ethylene glycol) diacrylate (PEGDA)-based materials and gelatin methacrylate (gelMA). Acrylation modification to PEG and gelatin render the material photosensitive for printing [43,44]. Combinations of already self-assembling proteins, such as keratin or decellularized extracellular matrix (ECM), with photoinitiators have allowed the cross-linking of soft hydrogels [45]. This method has the advantage of excellent structural integrity because no artificial interfaces result from droplets (inkjet printing) or lines (extrusion printing). Also, the electromagnetic spectrum and its multiple energy wavelengths allow for a broad range of chemistry alterations; various wavelength lasers, UV sources, and the visible light spectrum are common energy sources used in this technique. The use of light with all available intensities and wavelengths results in very fast and precise builds. Resolution of this technique ranges from 25 to 200 μm for commercially available printers [3,45] down to ~10 μm for two-photon polymerization setups [46]. However, because the material must be photosensitive, many biomaterials cannot be used or they must be chemically modified for photopolymerization [45,47]. A photopolymerizable material such as PEGDA is not in itself good for cell viability. Additional surface modifications are necessary to allow for cell attachment and material degradation by incorporating peptide sequences [48]. In addition, resolution is determined mostly by the laser spot size and therefore has high 3D resolution, but the prints often are warped because the mechanical properties are typically weak [3].

Overall, 3D bioprinting techniques vary in approach and can result in a wide array of medical applications from tissue repair to modeling disease. There has been tremendous progress in the development of the technique as well as the biomaterials synthesized to expand the palette of available 3DP bioinks. 3D bioprinting has exciting translational potential to produce implantable structures for regenerative medicine and high-throughput, reproducible drug screening. However, to realize this medical impact, researchers must continue to explore the architecture, the biocompatible yet printable materials, and the inclusion of proliferating and differentiating cells for fabricated living tissues to reach a desirable function.

BIOINKS

3D bioprinting may offer the potential to fabricate physiological tissue mimics; however, progress toward therapeutic application relies heavily on its integration with bioinks. Therefore, the development of biocompatible yet printable bioinks requires tremendous consideration to match physical and functional aspects of the desired tissue closely. Because 3DP technologies originally were designed for nonbiological applications, some of the materials used as inks for printing, such as thermoplastic polymers, ceramics, and metals, cannot translate to supporting living cells. Hence, one of the greatest challenges of the field is to find materials that are both biocompatible and printable.

As defined previously, printable biomaterials, or bioinks, encompass any printable material that (1) will interface with biological components during or after the actual print, or (2) is involved in the structural construction of scaffolds that will interface with biological components. The key difference between bioinks and other printable inks is cytocompatibility; bioinks cannot be toxic or produce any toxic by-products that could be detrimental to living cells, to the physiological function of the printed tissue, or to surrounding or down-stream native tissues.

Generally, materials used in the field of regenerative medicine are divided between natural and synthetic materials. Natural materials have all of the advantages of being physiological and inherently bioactive. However, natural materials lack tunability, batch-to-batch consistency, and often the physical properties necessary for printing. On the

TABLE 46.1 Bioink Materials Compatible With Associated Printing Techniques

	Material	Extrusion	Stereolithography	Inkjet	Sintering
Natural	Fibrin	[23,51]		[52,53]	
	Collagen	[23,54,55]	Multiphoton cross-linking [9]	[53,56]	
	Alginate	[6,7,14,18,23,57–60]		[61]	
	Hyaluronic acid (HA)	[62], Thiolated HA plus thiolated gelatin [63]	Acrylated HA [3]		
	Gelatin	[58,62,64,65]		[66]	
	Keratin		[45]		
	Agarose	[67]			
	Hydroxyapatite	[68]			[3,26]
	Carbohydrate glass	[11]			
Modified natural (semisynthetic)	Gelatin methacrylate	[69], Hybrid with gellan gum [70]	[44,57,71,72]	[73]	
	Methacrylated HA	[50]		[73,74]	
Synthetic	Poly(ε-caprolactone)	[7,20,21,75,76], Hybrid with starch [14,77], co-printed with polyurethane [60], hybrid with hydroxyapatite [78]			[27,29]
	Poly(glycolic acid)	[79], Hybrid with hydroxyapatite [78]			
	Poly(ethylene glycol) (PEG) or PEG-diacrylate	[80], PEG with reactive ends hybrid with multiple proteins [81]	[3,67,82–84]	[85]	
	Pluronic F127	[12,86]			
	Poly(propylene fumarate)	[22]	[10,87,88]		
	Poly(vinyl alcohol)	[89,90]	[3]		

other hand, synthetic materials benefit from a high degree of tailoring to specific physical property needs with inherent consistency to meet the printing technique's criteria. Still, synthetic materials often fail to match the biocompatibility of natural materials, and sometimes lead to toxic degradation products or the lack of cell-binding sites. Some groups have compromised the divided material set by synthesizing a semisynthetic class of materials such as gelMA or methacrylated hyaluronic acid [49,50]. Of our palette of biomaterials, only a subset is also suitable for bioprinting (Table 46.1).

For a bioink to be biocompatible as well as printable, the material must have the capacity to be accurately and precisely deposited with spatial and temporal control. Each bioprinting technique may require a different subset of material properties. For example, inkjet printing requires bioinks to possess low viscosity to avoid nozzle clogging; extrusion printing benefits from shear thinning properties to fluidize through the nozzle and quickly solidify once deposited; SLS must be able to become a fine powder and have an attainable melting temperature; and stereolithography requires photosensitive bioinks. These material properties to promote printability often come at the cost of compromising biocompatibility. As an example, a photosensitive material that cross-links in the presence of a photoinitiator that can be highly cytotoxic. Therefore, available bioinks are chosen to meet the demands of the particular printing process, but also for its ability to shield encapsulated cells from a possibly harmful printing process.

The requirements for printing depend on a variety of properties, including rheological behavior, the gelation process, or available biological interactions. From a rheology perspective, only specific ranges of viscosities match well with either inkjet or extrusion printing, but shear thinning is an example of a rheological property ideally suited for extrusion. The gelation process, or cross-linking, can greatly influence the geometric integrity of the print. Gelation can occur through ionic, thermal, enzymatic, or photocross-linking mechanisms; these ultimately dictate the printing technique with which the bioink is compatible. Biological interactions might need to be enhanced, especially for synthetic materials, by incorporating cell binding motifs or inclusion of an additional natural material.

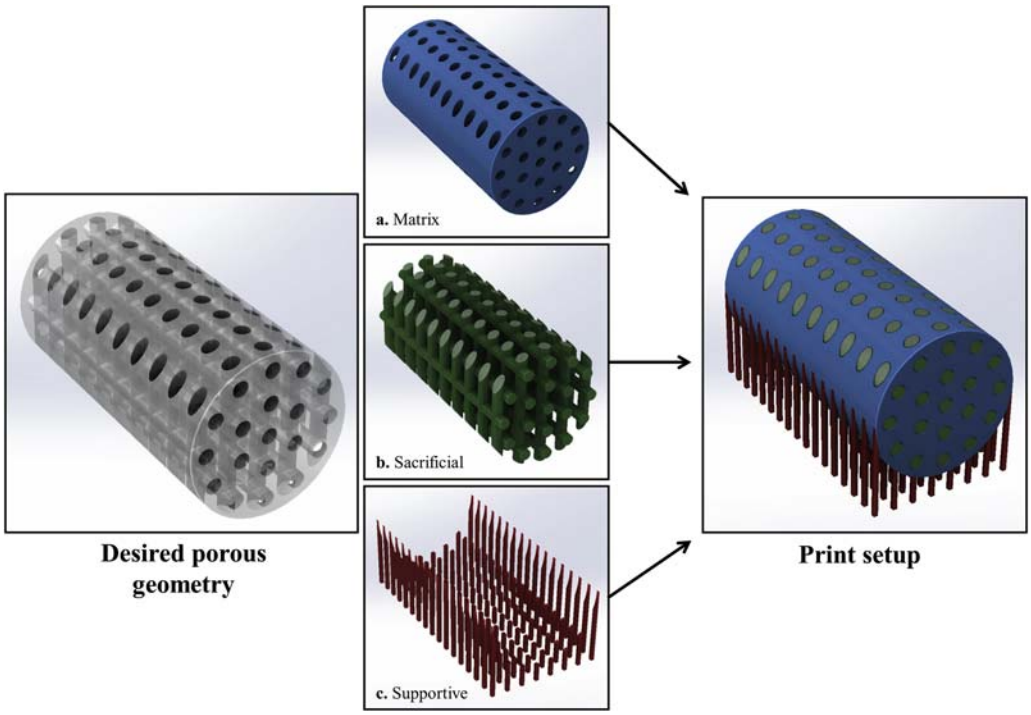

FIGURE 46.2 **Bioink categories** (*from left to right*): A desired final geometry volume can be fabricated with three different bioink approaches to result in the ultimate final print. The print setup shows how different bioinks would be incorporated during the print fabrication.

We categorize the available bioinks into three categories: (1) matrix or matrix-mimicking, (2) sacrificial, and (3) support [1]. As seen in Table 46.1, the same bioink can fall into several bioprinting strategies even if the bioink is used in a different way. Each category requires specific workflows, but they are ultimately brought together in the printing process, as illustrated in Fig. 46.2.

Matrix or Matrix-Mimicking Bioinks

Matrix or matrix-mimicking bioinks are printed and remain part of the scaffold system. A scaffold can be printed and consist of the matrix bioink material only (acellular scaffold), the matrix material with its surface chemically altered during or after the printing process (functionalized acellular scaffold), or the matrix printed with a loaded cell population (cell-laden scaffold). In all cases, the matrix bioink is the material that provides the mechanical structure to which cells adhere, and which will then be used to enhance cellular communication, proliferation, migration, and differentiation, and ultimately determine the function of the system.

As a structural element in a biological environment, there is a delicate balance between achieving the rheological and mechanical properties needed to print a self-supporting structure and the eventual effects that the material may have on the biological development of the cellular component. This balance is specific to the properties and function ultimately desired for the printed sample, anything ranging from soft and porous hydrogels for the in vitro culture and assessment of cells [65,81,91] to very strong and durable scaffolds for in vivo bone regeneration [10,76,92,93]. The desired mechanical and biological properties of the matrices are nevertheless restricted by the capacities of the 3DP technologies and the bioinks associated with each method. As has been reiterated in the literature, synthetic materials can be engineered to provide strong scaffolds with tunable mechanical and chemical properties [76]. Nevertheless, these materials have been traditionally associated with low biocompatibility [91], complex and demanding manufacturing processes (high temperatures, high pressures, strong solvents, etc.) [34,94], very low degradation rates [76,77], and, in some cases, cytotoxicity or harmful by-products [94]. On the other hand, natural materials inherently provide the adequate biological cues that cells need for proper development. The perfect combinations of amino acid sequences, protein ratios, growth factors, and cytokines are found in natural materials from fauna (ECM combinations, collagen, elastin, fibrin, keratin, hyaluronic acid [HA], chitosan, etc.) and flora (alginate, agarose, agar, silk, etc.) [3,91,95,96]. However, natural materials are also associated with weaker mechanical

properties and high batch-to-batch variability [7,46]. Natural variability is unavoidable and perfectly defined manufacturing protocols for natural materials are virtually impossible. Thus, truly tunable properties are difficult to predict and the reason why results in studies using ECM usually differ in a case-to-case scenario. As will be discussed in the next sections, synthetic and natural materials cannot be defined by positive and negative characteristics; researchers have been working on mitigating the weaknesses of both, either by biochemically altering the individual materials or by implementing synthetic—natural hybrids or combined prints that use the strengths of each.

Synthetic Materials

As mentioned, 3D bioprinting is an adapted technology; the original patent filed by Hull in 1986 [2] proposed using the stereolithographic method to optimize prototype manufacturing of plastic parts for industrial applications. Since then, the processes have evolved and revolutionized the industry and have bled into many other manufacturing applications including bioengineering, regenerative medicine, and tissue engineering. The original Hull patent was intended for synthetic materials, named "U- curable materials," which could be processed as a "fluid medium capable of solidification in response to prescribed stimulation" [2]. This definition is technologically viable today and could be applicable to most modern bioprinting methodologies, even if the solidification is not via UV cross-linking. The materials have greatly evolved and new ones have arisen, allowing researchers to incorporate synthetic materials and printing technologies into biomedical and tissue engineering.

The greatest strength of synthetics is that the manufacturing processes are well-known and can be engineered to specific mechanical and biochemical properties [91,94]. Polymer engineering allows control over molecular weights and distributions, as well as cross-linking densities, which can be tailored to control mechanical properties such as yield stress and strain, ultimate stress and strain, and elastic modulus [94]. This tailoring can occur as part of the polymer synthesis process, but it can be further modified in the printing or postprinting processes with curing or cross-linking steps. In the end, robust mechanical properties can be used to sustain high loads or adequately respond to elastic deformation, which is ideal for the structural scaffolding components of biological constructs. The synthesis of tunable mechanical properties also means that synthetic materials can be used in multiple 3DP techniques (Table 46.1) and result in constructs with consistent macroscopic and microscopic definition. The pore distribution in a biological scaffold is an important parameter that will define the presence or absence of vascularization for oxygen, nutrient, and metabolic waste transport in tissue regeneration [93]. The superior print resolution and fidelity of synthetics have been widely explored to produce complex morphologies that may be applied as biomimicking scaffolds in regenerative medicine (Fig. 46.3) or as structural supports in co-printing applications, a concept that will be further detailed in following sections.

In theory, just as the synthetic print resins can be modified to facilitate the manufacturing processes, surface modifications can be implemented to allow better interactions with biologic components. However, the manufacturing and modification processes and variables are usually demanding and work in narrow ranges to achieve specific properties. Often, printing techniques will involve high temperatures, toxic organic solvents, or cross-linking agents, which renders them incompatible with living cells and biological materials such as growth factors and proteins that aid cellular function and survival [94]. Synthetic materials generally do not support cell adhesion without additional surface functionalization for adhesion ligands such as arginine-glycine-aspartic acid, which is widely identified as a binding motif for proteins such as fibronectin, osteopontin, and fibrinogen [45,91,94]. Even with consistent morphology control and compatibility with surface modification strategies, synthetic polymers do not innately mimic ECM, which remains its weakest characteristic for the clinical translation of synthetic bioprinted scaffolds [3]. Still, synthetic materials that are commonly used in bioprinting applications include poly(ethylene glycol) (PEG) and PEGDA, PCL, poly(D,L-lactic-*co*-glycolic acid) (PLGA), poly(L-lactic acid) (PLA), and PPF, among others.

PEG has long been used as a coating on medical devices to control host-immune responses or alter degradation rates in vivo [94]. Furthermore, PEG is commercially available in many physical (linear, branched, molecular weight variation) and chemical variants (diacrylated variant PEGDA) and is approved by the US Food and Drug Administration as a biocompatible material, which makes it a versatile polymer for bioengineering [81]. Here, biocompatibility, a broad term, means the material does not kill cells or induce an aggressive immune reaction, but it does not necessarily mean that it induces cell adhesion or proliferation. As for other synthetic materials, PEG lacks attachment sites that cells need to adhere to a substrate. PEG requires chemical immobilization of binding motifs to support cell adherence and stem cell differentiation [3,94]. These characteristics usually result in PEG being used as a secondary plasticizer component in bioinks: even more so, PEG is often modified with acrylate groups to create photopolymerizable PEGDA, a variation that is commonly used with extrusion or stereolithography approaches, and can be easily coupled with natural biomolecules for cell-laden bioinks [3,81].

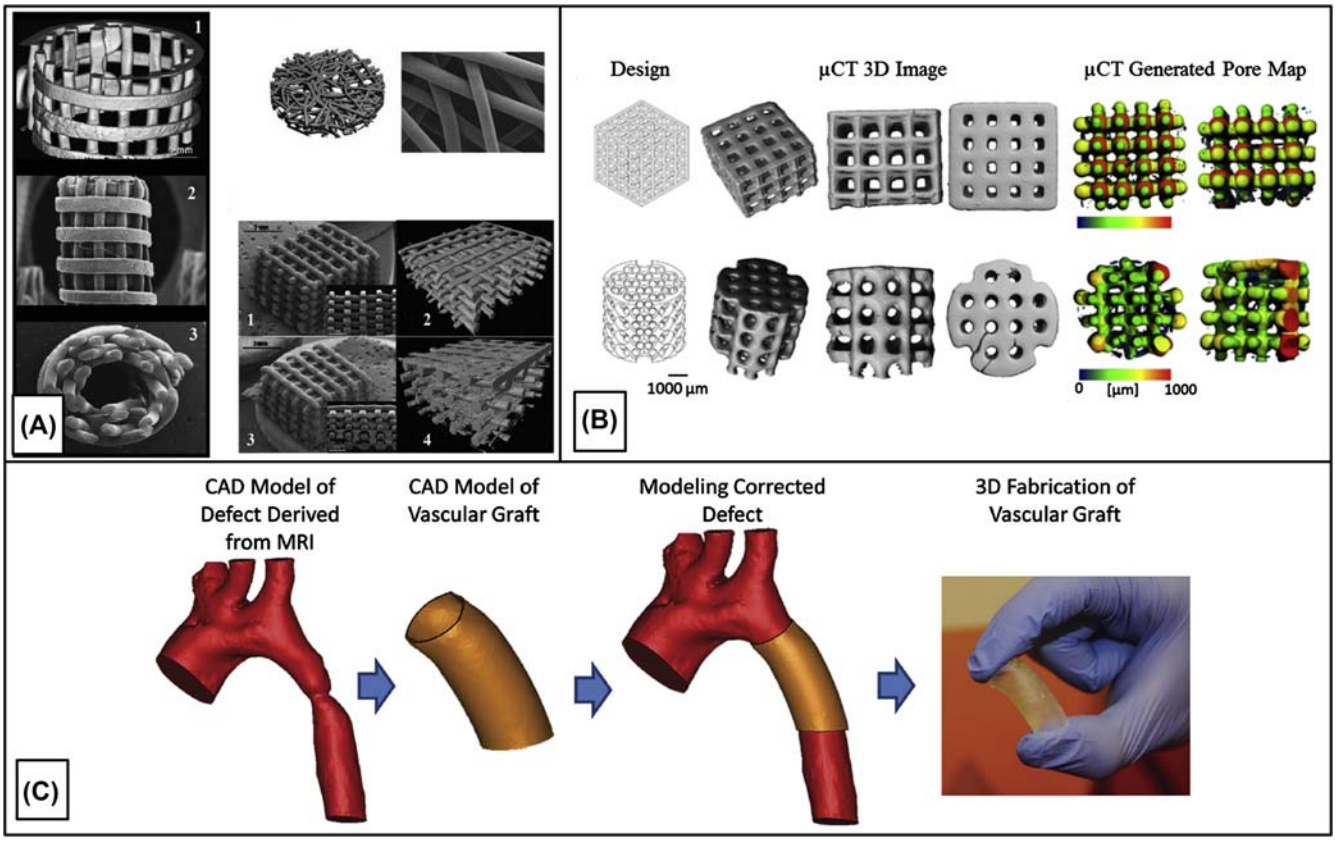

FIGURE 46.3 **Synthetic materials in bioprinting applications:** (A) Morphology of bioprinted starch- poly(ε-caprolactone) (PCL) scaffolds, including tubular scaffolds made by rapid prototyping and fiber-bonded scaffolds [77]. (B) Variations of three-dimensional (3D) printing (3DP) poly(propylene fumarate) (PPF) assessing scaffold and pore geometry to study the effects of pore geometry on cell viability and differentiation [10]. (C) Design and fabrication process of a 3DP PPF graft to treat a coarctation of the aorta [88]. *CAD*, computer-aided design; *MRI*, magnetic resonance imaging; *μCT*, microcomputed tomography.

PCL is synthesized by ring-opening polymerization of ε-caprolactone [77]. PCL is a high−molecular weight semicrystalline polymer that has good solubility, a low melting point, thermoplastic behavior, and an extended hydrolysis-induced degradation profile in vivo. This polymer is stable in the body for over 6 months [97]; then it exhibits nonenzymatic hydrolysis degradation of 2−4 years (depending on the molecular weight) at physiological pH and temperature, leaving no cytotoxic by-products [76,77,96]. Extended degradation is ideal for providing long-term load-bearing support during healing and regeneration processes [7]. PCL has a melting point close to 60°C, a relatively low temperature in the manufacturing industry, which allows easy processing [77,94,96]. Upon heating, PCL has viscoelastic properties ideal for the extrusion printing of constructs with elastic mechanical behavior, a characteristic that improves the brittle properties of polymers such as PLA and polyurethanes [97]. The Hutmacher group printed cylindrical scaffolds of medical-grade PCL−tricalcium phosphate by fused deposition modeling that required added growth factors to increase the osteogenic potential of seeded mesenchymal stem cells (MSCs) [76]. The growth factor−coated PCL scaffolds successfully completed up to 12 months of unrestricted load bearing in vivo within large tibial defects in sheep [76]. The use of additional osteogenic growth factors indicates the biochemical limitations of PCL; other than the hydrophobic nonspecific binding of cells, PCL lacks binding motifs that provide specific binding sites for cells [94]. Combination with natural materials or other functionalized materials is the usual approach to address this limitation. The Atala group, for example, concurrently prints PCL with hydrogels based on gelatin, HA, or fibrin; the hydrogels provide the biochemical cues for cellular adhesion and viability [94]. Starch, a natural polysaccharide, has also been widely used to improve the biocompatibility of PCL [77]. PCL−starch prints can enhance and stimulate osteoblast proliferation for bone regeneration, support hippocampal neurons and glial cells to treat spinal cord injury, or support bovine articular chondrocyte adhesion and proliferation, and glycosaminoglycans for cartilage tissue engineering [77].

PLA is a well-known established aliphatic polymer used in temperature-based extrusion methods [96,97]. It has a melting temperature close to 175°C, so it can be extruded in melt-based systems between 200°C and 230°C [97]. However, PLA glass transition occurs around 60°C and easily interacts with many plasticizers and solvents to change viscosity, a characteristic that allows printing at lower temperatures [96]. The resulting mechanical properties are usually high, with an elastic modulus around 1.5−2.7 GPa, but it tends to be brittle [96,97]. PLA is commonly used in orthopedic implants and drug delivery systems owing to its biocompatibility and biodegradability [96,97]. Nevertheless, its degradation is via the hydrolysis of ester bonds, which releases acidic by-products; in vivo, this may cause the localized decrease of pH through the release of lactic acid, inflammation, and cell death [97].

PLGA is the copolymer of lactide and glycolide, obtained via ring-opening polymerization, synthesized to address individual limitations and uncontrolled degradation of PLA and poly(glycolic acid) (PGA) [96]. Popular polymerization of D- and L-configurations of lactide yield the PLGA variation, which is frequently used owing to its improved toughness and easy manipulation of hydrolysis-driven degradation rates [96]. The Shah group synthesized "hyperelastic bone," a particle−laden 3D bioink that combines hydroxyapatite, a highly bioactive ceramic, and either PCL or PLGA [78]. The extrusion-printed structures exhibited mechanical and physical properties that allow further manipulation (sheets that can be rolled, folded, or cut). The hybrid with PCL showed highly elastic properties capable of reaching a 61.2% ± 6.4% strain and a tensile elastic modulus of 10.3 ± 1.3 MPa, a behavior superior to that of the PLGA combination (36.1% ± 4.3% strain and 4.3 ± 0.4 MPa elastic modulus). In terms of cell interaction, the PLGA combination showed better results; both the hydroxyapatite−PLGA and hydroxyapatite−PCL scaffolds supported human MSC adhesion and proliferation and induced osteogenic differentiation in the absence of engineered growth factors after 28 days [78].

PPF is a biodegradable polymer broadly applied in tissue engineering owing to its ability to form cross-linked networks through its carbon−carbon double bond [10,22]. The Mikos [22] and Fisher [10,88] groups extensively studied PPF and its cross-linking capabilities for 3DP and tissue engineering. Because it is biocompatible and can be photocross-linked, PPF is a prime candidate for 3DP via stereolithography [22,88], but it can also be as a viscous bioink for extrusion and cured using a UV source [22]. In the first case, the printing process is driven by the intensity of the light source and the proportions of photoinitiator and photoinhibitor in the bioink, but the resulting mechanical properties of the constructs heavily depend on the amount of printing or postprinting exposure to UV, which determines the polymer cross-linking density [10,88]. In the case of extrusion, PPF resins exhibit shear thinning behavior and the concentration of PPF drives the viscosity level. Other factors such as fiber spacing during deposition and pressure affect the pore size and fiber diameter, respectively, but interplay among the factors can also alter scaffold architecture [22]. Melchiorri et al. reported that human umbilical vein endothelial cells and human umbilical vein smooth muscle cells were seeded on stereolithography-printed PPF surfaces and proliferated in a 7-day study [88]. Similarly, MSCs were cultured on PPF scaffolds over 7 days and exhibited levels of metabolic activity that were not statistically different from cells cultured on standard tissue culture polystyrene [10]. In vivo, using 3DP PPF grafts to treat a coarctation of the aorta in mice for 6 months, printed PPF experienced a 40.76% ± 8.37% decrease in mass, and full endothelialization of the inner lumen on the grafts was observed even without preceding cell-seeding or surface modifications [88].

Natural Materials

Based on the definition earlier proposed, bioinks will interact with biological components (e.g., tissues, cells, proteins, growth factors) during or after the actual print or will serve as structural components during the printing of scaffolds that will interface with biological components. Without considering the specifics of in vivo or in vitro applications, there is an imminent interaction between cells and tissues with the bioink or its by-products. Natural materials are taken from animal or plant sources; these are materials that naturally developed to sustain cellular life cycles, nutrient and waste transport, and healing processes. They are composed of the perfect combinations of amino acid sequences, protein ratios, growth factors, and cytokines, thus intrinsically providing safe and nurturing interactions with cells. The composition provides the proper biochemical environment for cells to adhere or feel attracted to, subsequently allowing individual cells the healthy completion of the cell cycle and then induce cellular proliferation, migration, and differentiation. This is the basic definition of cytocompatibility, and natural materials provide a high intrinsic level of it [3,91,94]. Just as important, the composition and biochemistry of a natural material are designed to be degraded by physiologically viable processes through natural enzymatic and chemical processes, and to be discarded by natural metabolic activity, leaving behind no significantly harmful by-products [91]. Bioinks

from these materials can be further biochemically enhanced by encapsulating tissue-specific growth factors, genes, and other controlled-release chemical-regulation factors. Similarly, the surfaces of printed hydrogels can be functionalized by adding the same biochemical factors with both approaches, aiming to recreate environments more like those of in vivo tissues [78,91,94,95]. The balance between cytocompatibility and degradation means that these materials naturally go through the proper cycles and rates needed to induce healthy integration with host tissue [3].

Natural materials can be used as isolated, purified proteins (e.g., collagen, fibrin, keratin, or elastin) or as the natural protein combinations already present in the ECM, combinations that are specific to each type of tissue and determine the type of cells present, the bulk mechanical properties, and its function. Methods to obtain and alter natural materials in laboratory mainly consist of enzymatic cleaving, ionic interactions, and variations in temperature and pH [3]. These methods are used to cross-link the bioinks via multiple 3DP techniques (Table 46.1) and produce hydrogels with theoretically fine-tuned biochemical and mechanical properties. As it will be discussed further (see Cell-Laden Bioinks section), encapsulated cells or those that later migrate into the printed scaffolds are greatly affected by the mechanical cues imparted by the surrounding material. Cellular adhesion, morphology, migration, and especially differentiation, have been widely proven to be affected by the stiffness of the substrate [70,94]. In general, 3DP natural materials result in weak hydrogels difficult to manipulate into specific ranges of physical properties; they are limited by the inefficient or low-energy cross-links achieved by the traditional methods mentioned [94]. Weak mechanical properties have been used as an advantage when attempting to model or regenerate soft tissues or substrate for cell culture, but they are a severe disadvantage when the applications relate to load-bearing hard (e.g., bone, cartilage) or elastic tissue (e.g., muscle, skin, vascular and gastrointestinal tissues, ligaments, tendons). This major weakness has led to combinations with strong and elastic synthetic materials by co-printing or as hybrids, as will be detailed later (see Co-printing and Hybrid Bioinks section).

The intrinsic biocompatibility of natural materials is the main reason why these have been used to formulate bioinks for use in a wide range of in vitro and in vivo bioengineering and regenerative medicine applications (Fig. 46.4). The most popular materials are generally proteins from mammalian origin such as collagen, gelatin and gelMA, fibrin, HA, elastin, and keratin; similarly, popular polysaccharides from plant sources include alginate, starch, agarose, and silk, among others.

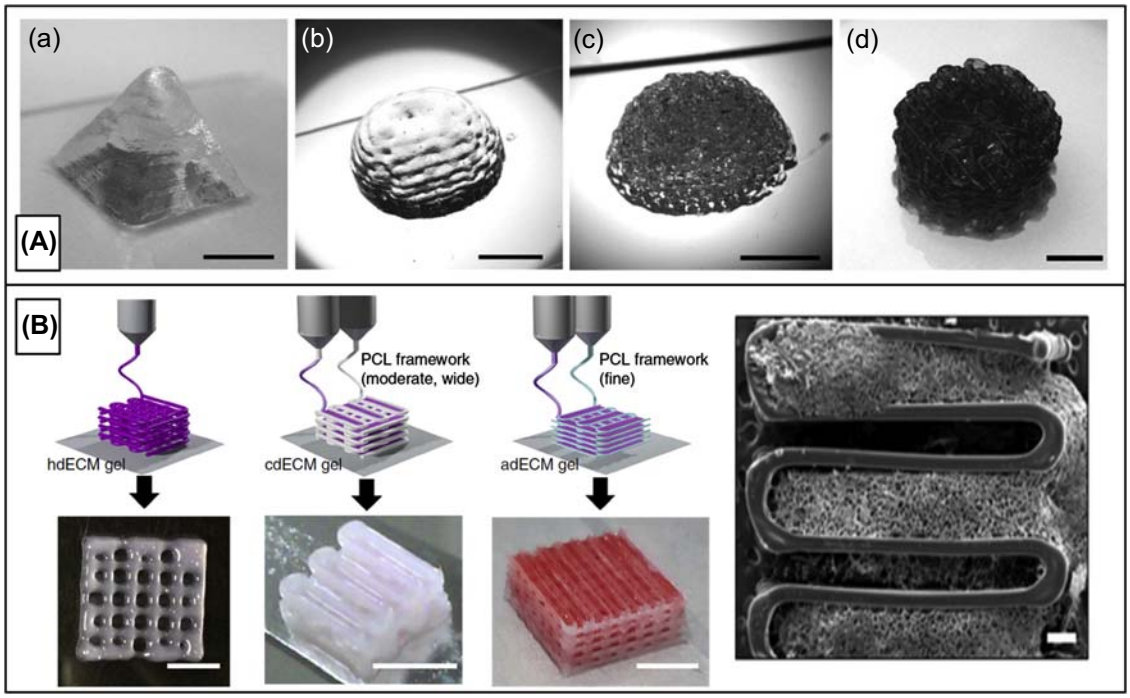

FIGURE 46.4 **Natural materials in bioprinting applications:** (A) Examples of plotted gelatin methacrylate–gellan structures after UV curing: (*clockwise*) solid pyramid, solid hemisphere, porous hollow cylinder, and 30% porous hemisphere (5-mm scale bars) [70]. (B) Examples of decellularized extracellular matrix (dECM) printing in combinations with polycaprolactone (PCL) framework, including heart dECM (hdECM), cartilage dECM (cdECM), and adipose dECM (adECM) (scale bar = 5 mm). Also, scanning electron microscopy of PCL tissue constructs with adipose-derived dECM bioink (scale bar = 400 mm) [100].

Collagen, particularly type I collagen, is the most abundant ECM protein in tissues [9,94,98]. The most common types of collagen are the fibril-forming collagens (e.g., type I, II, III, V, and XI); they are the main component in the ECM of tissues such as bone, tendon, ligament, skin, muscle, or cornea [9,98]. Because of its natural abundance in all types of tissues, collagen has variations that interact with most types of cells and it performs a wide range of mechanical roles in soft, elastic, or hard tissues. For this reason, collagen is arguably the material that most researchers have tried to adapt for bioengineering applications and has been reported as the most commonly used for cell and tissue culture [94]. It has been used in 3DP techniques such as extrusion [23,55], stereolithography [9], and inkjet [51], mostly employing variations of pH-triggered or temperature-triggered gelation [98] that range from hours to minutes [91]. Cross-linking of collagen by altering pH using solubilized sodium bicarbonate solution has been used to construct multilayered cell−hydrogel composites. This method provided a novel approach to printing both fibroblasts and keratinocytes in a single experiment to model dermal−epidermal-like distinctive layers in a 3D hydrogel [15]. Natural material hydrogels have typically been reported to have subpar printing resolution compared with synthetics. Nevertheless, Bell et al. reported printing line widths of about 1 μm using multiphoton cross-linking of type I collagen with a flavin mononucleotide photosensitizer, which confers structural control at a microscale level [9]. Collagen has also been widely used in combinations with other natural and synthetic materials, principally bringing strong biocompatibility to the mixture [13,91,99].

Gelatin is the denatured form of collagen that has undergone partial hydrolysis [9,94]. As collagen, gelatin is characterized by its wide availability, biocompatibility, predictable enzymatic degradation, nontoxic by-products, and inherent cell binding motifs [70]. It has been involved in engineering soft and hard tissues ranging from liver to bone, either by itself or as part as hybrids such as gelatin−alginate, gelatin−fibrin, and gelatin−HA [95,100]. Gelatin is widely regarded as the easiest protein to print, mainly because of thermally responsive behavior that allows extrusion at temperatures below 20°C and hydrophobic cross-linking [64,94]. However, the melting temperature of gelatin (30−35°C) is below physiological temperature, which severely limits its clinical application in vivo [95]. Even with the high resolution obtained by extrusion-based printing [70], gelatin hydrogels are usually soft and limited by temperature, which requires further cross-linking either by postprinting approaches (e.g., using glutaraldehyde or thrombin [95]) or by adding functional groups [94]. Like the acrylate modification on PEG that produces the versatile PEGDA, methacrylamide photoinitiator groups can be used on gelatin to obtain gelMA to produce a photocross-linkable resin. This modification enables irreversible cross-linking, generally by UV irradiation, that preserves printed architectures under physiological conditions [70]. UV exposure time and gelMA concentration regulate printability, whereas the degree of methacrylation determines the mechanical properties and additional acetylation can be used to influence the rheological properties of the bioink further [70,96].

Fibrin is a glycoprotein composed of fibrinogen monomers; it is synthesized in the liver by hepatocytes. In the body, it has important roles in blood clotting and wound healing [94]. The clotting pathway has been replicated as a cross-linking method for 3DP; thrombin is used to polymerize fibrinogen rapidly into cross-linked fibrin [12,94]. As a glue-like gel, fibrin has been used clinically as surgical hemostatic agents and sealants [94]. Enzymatically quick cross-linking rates have been exploited with extrusion and inkjet-based printing [51,52,94], but the mechanical properties of the constructs have been paradoxically described as both robust [94] and weak regardless of the concentration of the reagents [46]. Fibrin-based hybrids materials with natural or synthetic components are usually reported to fine-tune mechanical properties depending on the application, including cross-linking with PEG and adding PGA fibers, PLGA, hydroxyapatite, or demineralized bone matrix [46].

Alginate is a natural polysaccharide derived from algae or seaweed. Sodium alginate is generally cross-linked in calcium chloride ($CaCl_2$) aqueous solution, via an ion exchange reaction between sodium and calcium [3,101]. This chemically efficient reaction results in biocompatible, low−polymer density, high−water content hydrogels [3]. Traditionally, cell encapsulation in calcium alginate hydrogels was the main application of alginate in tissue engineering and bioengineering models, despite the controversial effects of $CaCl_2$, the cross-linking reagent, as well as sodium citrate and ethylenediaminetetraacetic acid (EDTA), commonly used chelators, on cell viability [94]. This ionic cross-linking approach has been implemented in bioprinting. It works particularly well in extrusion-based systems that extrude alginate resin into $CaCl_2$ reservoirs [6,7,14,18,60]. Cells can be suspended in a solution of sodium alginate in cell-specific culture medium, after which cross-linking is induced by incubation in $CaCl_2$ and results in a hydrogel construct laden with cells. This approach has been successful in bioprinting, such as in human cardiac-derived cardiomyocyte progenitor cells (hCMPCs) for an in vitro committed cardiac tissue [18]; heterogeneous scaffolds with MSCs and chondrocytes (in alginate with osteogenic or chondrogenic differentiation medium, respectively) for osteochondral tissue engineering [6]; or encapsulated HepG2 liver cells printed directly on a polydimethylsiloxane chamber for a microfluidic pharmacokinetic liver model [101]. Nevertheless, Carrow et al. stated that there were major challenges for bioprinting alginate: (1) the difficulty of controlling the ionically driven process,

which results in unpredictable microstructures; and (2) the high solubility of alginate, which was a disadvantage when printing thick structures by extruding directly into $CaCl_2$ aqueous solutions [96].

Hyaluronic acid (HA), also called hyaluronan, is a hydrophilic nonsulfated glycosaminoglycan present in the ECM of tissues [94]. The Atala group has regularly used HA in bioprinting processes by adding photocross-linkable methacrylate groups that can undergo free radical polymerization when irradiated with UV light. This modification allows soft hydrogels to be printed via stereolithography or extruded with additional postprinting UV curing [62,63,94]. Although not mechanically robust on their own, HA hydrogels have served in cutaneous and corneal wound healing, prototype vessel structure bioprinting, tumor modeling, and 3DP of cell-laden structures [94].

Other lesser-used natural materials for bioprinting include proteins and polysaccharides such as elastin [98], keratin [45], starch [77], and agarose [3,91]. Despite the success of using isolated natural polymers, there has been growing interest in using the innate combination of proteins in the ECM. The ECM allows structural support and anchoring to cells and also provides a substrate for transport and communication, ultimately affecting the survival and differentiation of cells. Cell−ECM interactions are extremely complex and cannot be fully and precisely replicated in vitro or engineered from isolated proteins. Several groups have presented decellularized ECM (dECM) bioinks derived from adipose, cartilage, heart, bone, or skin tissues. The combination of proteins in the ECM can be understood as a hybrid of multiple natural materials; therefore, available cross-linking methods and bioprinting approaches have been successful in producing 3D dECM scaffolds. Pati et al. developed dECM bioinks that can be extruded as filaments; printed scaffolds can then undergo gelation at physiological temperatures, remaining in the solution below 15°C and cross-linking by incubation at 37°C [100].

Co-printing and Hybrid Bioinks

Approaches attempting to print synthetic and natural materials individually have produced scaffolds with mechanical and biochemical properties that affect cells and tissues differently, and thus can be used in different types of in vitro and in vivo applications. A common generalization in the field is that synthetics are used for their strong, finely tunable mechanical properties; nevertheless, it has been proven that they can provide tunable degradation rates, functionalization capabilities, and various degrees of biocompatibility and print resolution. On the other hand, natural polymers have proven to be highly compatible with a wide variety of cells and biological components, mostly owing to their inherent composition and function. As with synthetics, it is hard to generalize the negative characteristics of natural bioinks, but the properties (mechanical or biochemical) are rarely fine-tunable and usually are presented as ranges and wide error margins commonly associated with batch-to-batch variability. Overall, natural material properties, printing quality, and in vitro or in vivo behavior can be described as unpredictable and difficult to replicate. Combining both types of materials has been an increasingly popular hypothesis that relies on the positive properties of each. In theory, synthetic materials provide structural integrity and printing definition, whereas natural polymers can be used to incorporate cells and other biological components [95,97,100]. Two broad categories for combining synthetics and natural materials as bioinks for 3DP applications are (1) co-printing, the individual but parallel printing of natural and synthetic resins; and (2) hybrid bioinks, in which the resin is a uniform solution of both materials printed as a single construct.

Co-printing approaches rely on printing synthetic scaffold structures with robust mechanical properties onto which natural hydrogels can be printed. This addresses the common limitation of natural materials, the inability to maintain uniform 3D structures in vivo (e.g., to allow tissue load bearing or provide a specific porosity or microstructural pattern) or in vitro (e.g., to be handled robustly in bioreactors, or as cell substrates), by integrating a synthetic scaffolding [14].

The main challenge is that co-printing relies on technologies that dispense more than one material during the printing process, sometimes with radically different deposition necessities and cross-linking mechanisms, as illustrated in Fig. 46.5. In extrusion-based systems, for example, the rheology of the materials is the driving principle, and variables such as viscosity, flow rate, temperature, and pressure determine the extruded line width, fabrication time, or print resolution [7]. Shim et al. used a multihead tissue−organ building system possessing six dispensing heads to dispense thermoplastic PCL and alginate hydrogel individually in the same structure, to produce constructs containing two different cell types for osteochondral tissue regeneration [7]. As they reported, the viscosity of alginate solution was about 10 Pa s and needed low driving forces but high force control to achieve high resolution; on the other hand, viscosity for PCL ranged from 1020 to 2560 Pa s at 80−120°C (a temperature high enough to damage cells) which required high driving forces to extrude [7]. The same PCL−alginate approach was reported using a multihead deposition system, they printed PCL and chondrocyte-laden alginate with and without transforming growth factor β [14]. Here, PCL was extruded at 80 mm/min at 80°C using a 650-kPa pneumatic pressure. Sodium alginate was deposited at room temperature between lines of PCL at 400 mm/min, and then cross-linked in sodium chloride

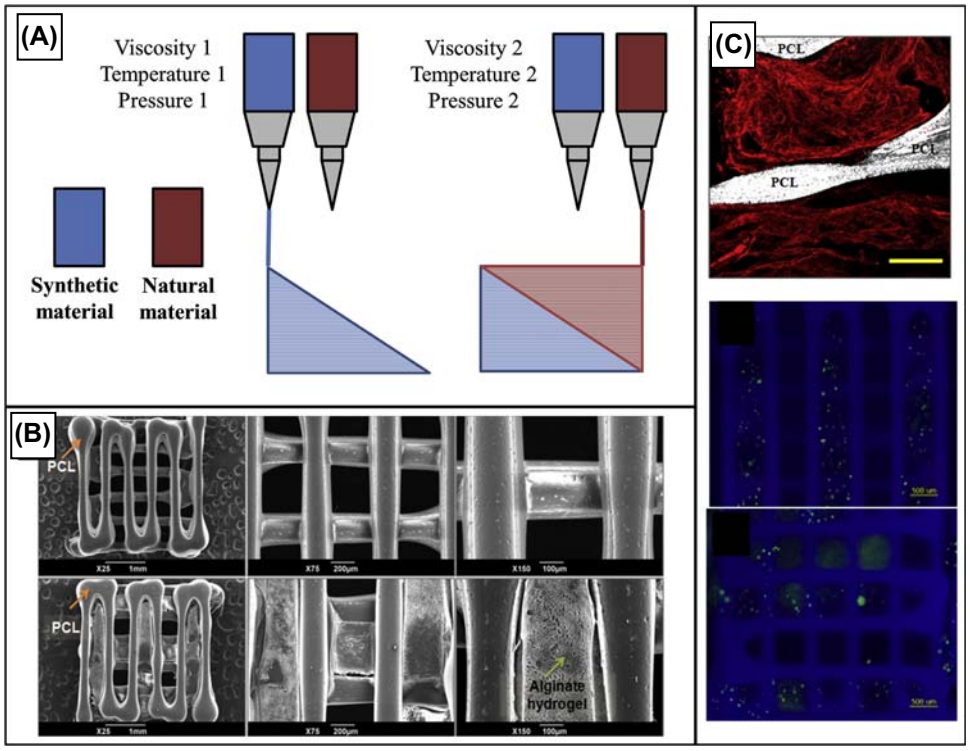

FIGURE 46.5 **Co-printed systems with synthetic and natural bioinks:** (A) Synthetic and natural materials are deposited using independent cartridges and print heads. The different properties of each material can be exploited to print them separately and sequentially, but it is necessary to consider force and temperature shocks that may alter one material when the other is printed in contact with it. (B) Bioprinting of poly(ε-caprolactone) (PCL)-alginate scaffolds. Scanning electron microscopy images comparing PCL and PCL−alginate scaffolds [14]. (C) AlexaFluor 594 red fluorescent dye positively staining type II collagen fibrils deposited by viable chondrocytes encapsulated in the same PCL−alginate scaffolds. Also, live/dead assay of chondrocytes showing cellular viability on the natural material but none on the synthetic component [14].

[14]. These cases illustrate the complexity of printing two different materials in the same structure; as stated before, the key lies in the independent extruding heads, in which the variables of the process (temperature, speed, pneumatic pressure, architectural patterns, etc.) can be controlled independently for each different material.

The ability to control each material separately but still building a single construct has high impact in resolution. In particular, the ability to place cells and materials with different properties into specific patterns confers high control over the resulting mechanical and biochemical behavior of the whole construct. Complex tissue constructs have been achieved via co-printing, such as the muscle−tendon unit (MTU) approach reported by the Atala group [60]. The MTU is the interface between muscle, which is elastic and fibrous in nature, and the tendon, which is stiff and sparsely cellular. In this approach, natural hydrogels were composed of gelatin, HA and fibrinogen. Elastic polyurethane and hydrogel laden with C2C12 myoblasts were chosen for the muscle side, whereas stiffer PCL and National Institutes of Health (NIH)/3T3 fibroblasts gels were selected for the tendon group [60]. The co-printing approach allowed the controlled construction of the interface in which two mechanically different tissues with different cell populations flawlessly met (Fig. 46.5). They reported that the construct was not only able to mimic the complex mechanical behavior of the MTU but successfully retain cell viability in both hydrogel portions (C2C12 cells with 92.7% ± 2.5% viability and NIH/3T3 cells with 89.1% ± 3.3% after 7 days [60]). The PCL-hydrogel co-printing approach has also been used to mimic mandible bone, ear cartilage, and skeletal muscle for tissue engineering [92].

Not restricted to producing tissue scaffolds for eventual in vivo applications, co-printing enables the construction of complex models for in vitro testing, particularly vascularization, microfluidic, and tissue-on-a-chip models. Having multiple heads depositing various materials and cells, under strict spatiotemporal control, has allowed researchers to produce highly complex models that more closely resemble the behavior of biological systems in vitro. These efforts usually require the use of cell-laden hydrogels (see Cell-Laden Bioinks section) and complex types of bioinks such as sacrificial (see Sacrificial Bioinks section) or supportive bioinks (see Supporting Bioinks and Supporting Baths section). An interesting example of such structures can be observed in the work of the Lewis group. Those researchers used the co-printing of natural and synthetic materials to develop in vitro models of tissues

and vascularization [12]. They concomitantly printed natural materials that included cell-laden castable and printable ECM composed of fibrinogen and gelatin, cross-linked via thrombin and transglutaminase enzymatic reactions, whereas the synthetic parts included silicone chip bases and Pluronic sacrificial materials [12]. These materials were deposited and cross-linked independently and sequentially to produce a highly organized vascularized tissue analogue based on the strong characteristics of both natural and synthetic materials.

Hybrid bioinks are the second approach to integrating synthetic and natural materials. In this case, there is a single bioink solution that includes both types of materials as solutes. Generally, hybrid bioinks are composed of a synthetic substrate solution with specific mechanical and rheological properties into which a natural component is mixed to alter biochemical and biocompatibility properties, as illustrated in Fig. 46.6. For synthetic materials, adding natural groups to the bioink usually results in improved compatibility with cellular processes, including binding sites and growth factors or reducing the high hydrophobicity of synthetics [14]. For natural materials, the benefits are usually observed as structural or mechanical, but the inclusion of synthetic polymers to the protein chains also enables natural materials to be processed using the techniques and equipment designed for synthetics. The weak ionic interactions or unpredictable enzymatic processes reserved to process alginate, fibrin, or collagen can be changed for optimized and finely tunable techniques such as photocross-linking or high-resolution extrusion [94].

The hybridization of the materials can be achieved by mechanical entanglement of the materials in solution or by chemically joining the polymer and protein chains. The first is a common approach to improving the mechanical or rheological properties of natural materials. Narayanan et al. used human adipose tissue stem cells loaded in alginate bioink with suspended PLA nanofibers [102]. The cell-laden alginate solution could be prepared separately from the nanofibers but they were vortexed together into a single solution, printed, and cross-linked, trapping the PLA within the hydrogel with no cross-linking interaction between the two. This approach was successful in producing constructs that allowed stem cell differentiation down the chondrogenic pathway; more interestingly, it revealed a

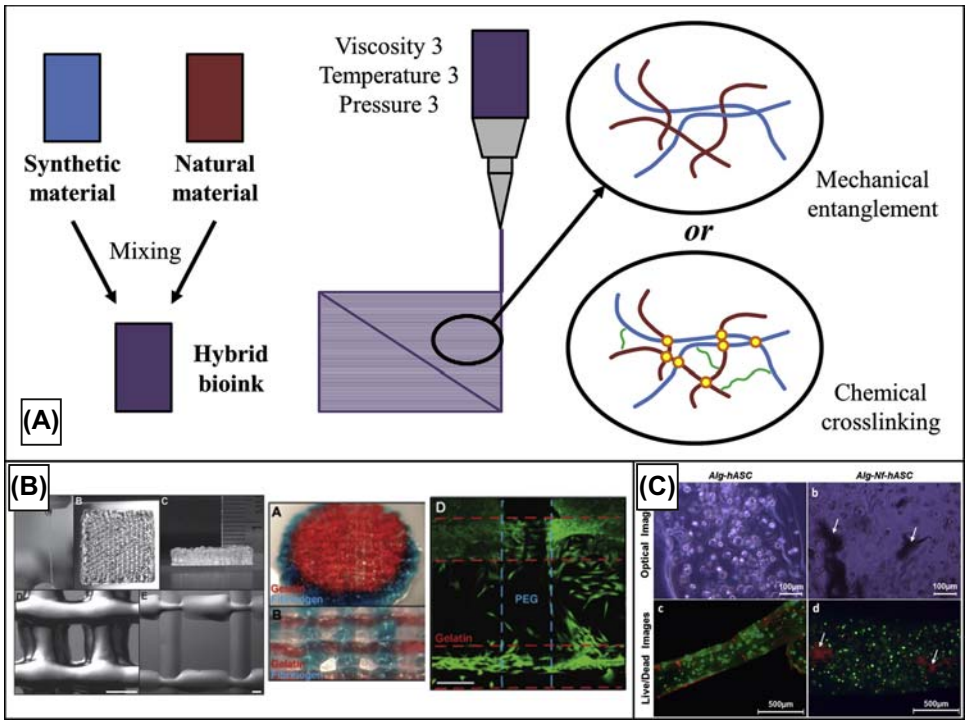

FIGURE 46.6 **Hybrid bioinks combining synthetic and natural biomaterials:** (A) Hybrid bioinks can be three-dimensionally (3D) printed from a single ink containing both the synthetic and natural components. The two materials can be printed into a single construct and structurally held together either by mechanical entanglement of the polymers or by biochemical cross-linking (a process that can occur during resin formulation or after printing). (B) 3D bioprinting of poly(ethylene glycol) with reactive ends (PEGX)—biomolecule hybrids. Extrusion printing of PEGX—gelatin hybrid. Next, PEGX—gelatin (*red*) and PEGX—fibrinogen (*blue*) can be printed in a single cylinder. Finally, PEGX—poly(ethylene glycol) (PEG) and PEGX—gelatin fibers crosshatched, live/dead assay show viable human dermal fibroblasts preferentially adhering to PEGX—gelatin, a synthetic—natural hybrid, but not to the PEGX—PEG, a synthetic mixture [81]. (C) Optical and live/dead fluorescence images (after 16 days) showing cell viability in bioprinted strands of alginate with adipose-derived stem cells (Alg-hASC) and of alginate-poly(L-lactic acid) (PLA) nanofibers with human adipose-derived stem cells (Alg-Nf-hASC) [102].

method to use the distribution and alignment of the PLA nanofibers to stimulate orientations within the ECM-mimicking hydrogels [102]. The second approach relies on chemically altering and cross-linking the synthetic and natural chains. It is commonly used to improve the biocompatibility of the synthetic portion or print the natural material using synthetic methodologies. The chemical modification allows the personalization and optimization of the resulting bioink chain, which means higher specificity of the printed materials to cell or tissue functions. The Shah group, for example, used functionalized PEG to include a variety of proteins in extrudable, tunable, and cell-compatible bioinks [81]. As illustrated in Fig. 46.6, PEG with reactive ends (PEGX) is used to bridge protein and polymer chains in a variety of configurations, producing mixtures such as PEGX−collagen, PEGX−gelatin, PEGX−fibrinogen, and PEGX−PEG, among many others, that can be successfully loaded with cells and printed via extrusion [81].

Cell-Laden Bioinks

Current definitions of bioinks refer to resins that are loaded with cells and printed. As described before, we expanded the definition of bioink to include several categories of printable materials, and do not necessarily consider cells to be the determinant "bio" factor. Nevertheless, the importance of cells for bioengineering and regenerative medicine is undisputable, and cell-laden bioinks are crucial for the development of 3D bioprinting technologies and the goal of printing functional in vivo and in vitro tissues and organs (Fig. 46.7).

Synthetic and natural materials have been proven to have various degrees of success in cell compatibility, tissue integration, and tunable mechanical and biochemical properties, so why incorporate the complex additional factor of cells? It is commonly accepted that the acellular scaffold approaches have poor translation in vivo, mostly owing to the limitation of cells adhering only to the surface of the constructs. The success of this approach is unpredictable, locations and concentrations of growth factors or chemoattractants within the constructs cannot be guaranteed, and cell behavior cannot be controlled [14]. We have mentioned before that the key term that defines modern bioprinting is control. Being able to control where cells, matrix, growth factors, and other biological components are placed results in structures with higher orders of specificity and functionality. If materials and cells can be located and properly stimulated to construct gradients, strata, or clusters, there is a higher chance for success without relying on the unpredictable colonization of native cells. Fedorovich et al. exploited this control feature to reproduce the specific spatiotemporal distribution of cells and ECM in osteochondral tissue [6]. The bioinks consisted of alginate solution in osteogenic or chondrogenic differentiation medium, in which MSC or chondrocytes, respectively, were added. After successful extrusion mimicking the adjacent bone and cartilage portions, ionic cross-linking, and subcutaneous implantation in mice, the dual, heterogeneous scaffolds showed two different cell lineage commitments, with each type of cell remaining in its printed position and depositing lineage-committed ECM [6]. Another multiphase approach to osteochondral tissue engineering was presented by the Demirci group, aiming to study tissue interfaces in the anisotropic composition of fibrocartilage [8]. Human MSCs were encapsulated in gels and printed by droplet deposition in an arrangement with zone-specific biochemical factors and ECM components (transforming growth factor-β1 for fibrocartilage and bone morphogenetic protein-2 for bone regions). Again, cells showed different lineage commitment by upregulating osteogenesis- and chondrogenesis-related genes defined by the position and matrix in which they were printed, yet constructing a single heterogeneous scaffold [8].

Cell-laden bioinks are generally hybrid or natural bioinks that can be 3DP into hydrogels. The materials provide innate cell-binding motifs, hydrophilic surfaces, and low cytotoxicity to promote cell adhesion [94]. The hydrogel structures provide soft, degradable, and swelling networks that mimic ECM and allow cell migration, metabolism, and differentiation with minimal restriction [70,97]. The mechanical properties of 3DP hydrogels can be modified by regulating cross-linking density, the linking chemistry, or polymer concentrations to match properties close to those of native ECM [94]. Structural properties of the microenvironment, such as stiffness or composition, can deliver biochemical cues by mechanotransduction to regulate cell shape, migration, and differentiation lineage selection [81,91,94]. As an example, bioinks used for high-resolution prints generally produce stiffer gels ideal from a 3DP and structural standpoint, but the high elastic modulus will drive stem cell differentiation toward the stronger tissue lineages (bone and cartilage), making it an obsolete approach to produce soft tissues [70].

Hydrogels seem to have the ideal characteristics for cell adhesion and sustenance, but the material−cell tandem must also work with 3DP methods and account for the impact of the processes on cell function after printing. First and foremost, no part of the bioink, printer setup, additional cross-linking mechanisms, or by-products can be cytotoxic; and they have to be sterile-compatible. This seems straightforward, but it considerably reduces the available materials and processes that can be used [3]. 3D bioprinting methods mostly rely on physical forces or temperature to deliver materials. The most popular methods, extrusion-based and inkjet printing, rely on some mechanism of pressure that pushes the bioink through a nozzle. This setup translates pressure on the cells first as a compressive

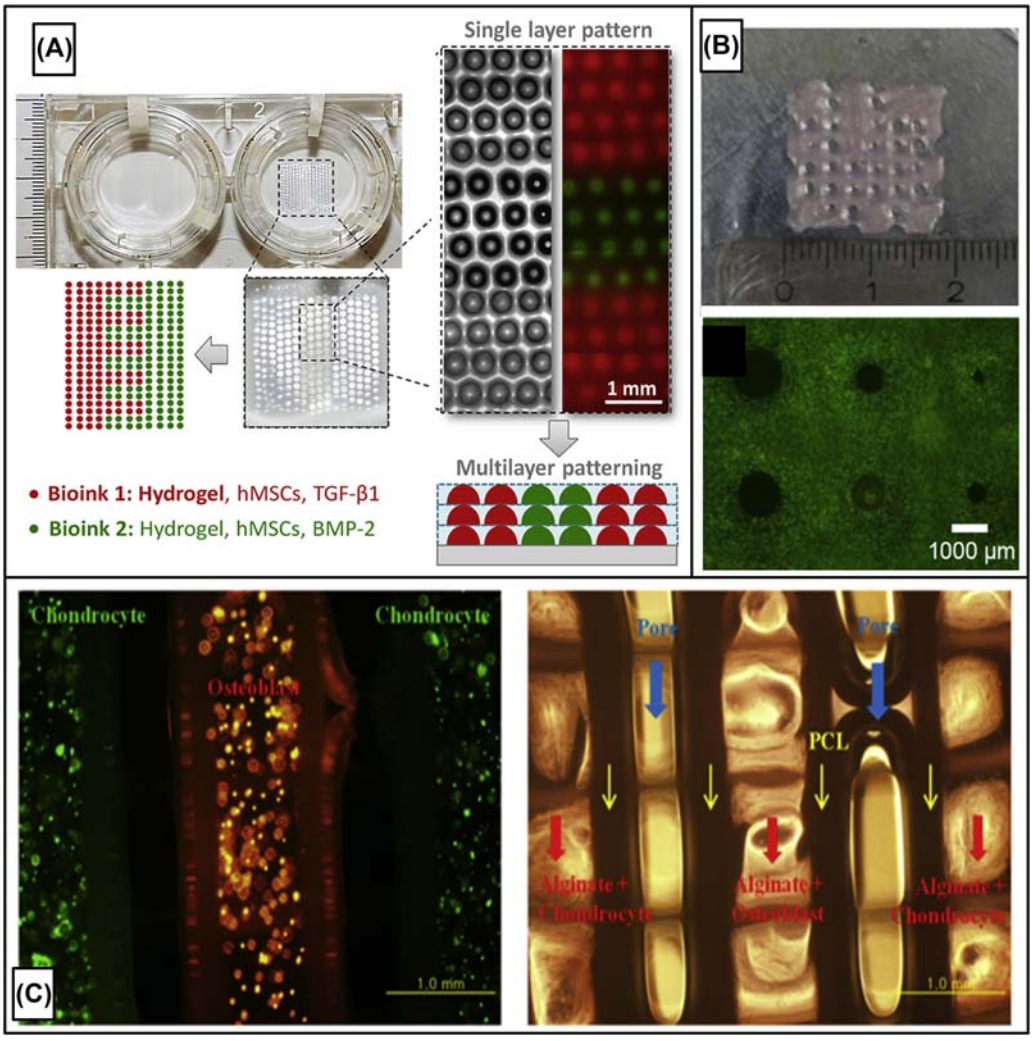

FIGURE 46.7 **Applications of cell-laden bioinks:** (A) Micropatterning and bioprinting the anisotropic three-dimensional (3D) fibrocartilage phase, composed by merging two human mesenchymal stem cell (hMSC)-laden gelatin hydrogels with either tendon or bone growth factors [8]. (B) Macroscopic and live/dead images of printed human cardiac-derived cardiomyocyte progenitor cells in 5% alginate scaffold [18]. (C) Fluorescence image of alginate hydrogels laden with chondrocyte (*green*) and osteoblast (*red*) cells and dispensed into a poly(ε-caprolactone) (PCL) framework. Every other pore is empty for oxygen and nutrient transportation [7]. *BMP-2*, bone morphogenetic protein-2; *TGF-β*, transforming growth factor-β.

force within the cartridge and then as shear stress while they are moving through the nozzle. Varying the pneumatic pressure, extrusion speed, and nozzle diameter regulates the stress delivered to cells and has been proven to affect cell viability during and after printing [14,94]. A variety of printing protocols have studied these parameters and successfully printed multiple types of cells and materials with a very high viability rate: extrusion of MSCs and chondrocytes in alginate with 89% viability 5 h after print [6] or 97% cell viability after thermal inkjet deposition [34]. High shear rates and shear stress have been proven to harm the cells, but high viability may be explained: (1) Stress causes protein denaturation by damaging the tertiary or quaternary structures of the chains, a process that is reversible with time [34]; or (2) natural or hybrid gels have shear thinning behavior, which decreases stress on the cells even at high shear rates or pressures [6,100]. Bioink viscosity and surface tension can also be modified to reduce shear stress using solvents or surfactants [34,95], although these must comply with minimum cytotoxicity requirements. In the case of stereolithography, the UV intensities required to initiate and sustain photoinitiator and photocross-linking reactions can negatively alter cell morphology and viability [3]. The amount of energy radiated can also cause irreversible damage to the cells as a result of increasing temperatures and dehydration.

Temperatures different from physiological 37°C, both high or low, and drastic changes will alter cell metabolic activity and may cause cell death [94]. This is a critical consideration for co-printing approaches, in which the

temperature required to deposit synthetic materials could easily be above 60−100°C and then solidify on cold surfaces. Both extremes having the potential for irreversible cell damage [4,6,12,14]. Chemically or pH-induced cross-linking by ionic and physical mechanisms can also harm cells. Additional cross-linking or postcuring approaches, such as postprint glutaraldehyde or EDTA chemical cross-linking or additional UV irradiation, which guarantee 3D geometries and mechanical properties, are commonly seen as additional negative factors [4].

A critical element to maintain cells in the bioinks is the permanent need for proper oxygenation and metabolic transport. In vivo, any cell will be located within 100−200 μm of a capillary, the maximum distance for adequate gas and metabolic exchange [4,7,91]. Thick-casted or printed hydrogels without pores do not allow diffusion deep enough to supply oxygen or nutrients and result in necrotic cells encapsulated in the center of the structure. Generally, the design of 3DP architectures is envisioned with pores or channels that provide open transport pathways and open vascularization channels. Interconnected pores, constructed by weaving strands or layered patterns, with dimensions ranging from tens to hundreds of micrometers usually allow transport through the scaffolds and report high cell viability and development [7,10,64,95]. Another common approach is to induce fast vascularization of the constructs, generally by adding endothelial progenitor cells or growth factors and using bioreactors [4,12].

Overall, accounting for the strengths and limitations of printing cell-laden bioinks, numerous approaches regularly report positive effects of this approach on cell behavior for a wide range of applications. As an in vitro example: Gaetani et al. printed a model of undifferentiated but committed cardiac tissue [18]. Here, sodium alginate was dissolved in a culture medium and mixed with hCMPCs. 3D constructs were obtained by printing strands into layers, stacking them to obtain different degrees of porosity, and cross-linking with $CaCl_2$. Compared with regular 2D cultures, bioprinting had no effect on cell viability and proliferation, but it increased cardiac lineage commitment by upregulating early and late cardiac transcription factors and markers [18]. On the other hand, in an in vivo application aiming to produce an implantable bioartificial liver, Wei et al. used a gelatin−fibrinogen matrix loaded with rat hepatic cells to produce 3D porous constructs via extrusion printing and thrombin-induced gelation [51]. After extrusion, about 98% of the hepatic cells were reported to be viable, steadily producing albumin, and dissolving the surrounding gelatin matrix throughout the culture time [51].

Sacrificial Bioinks

Sacrificial bioinks enable the fabrication of complicated structures and open geometries without dealing with many of the difficulties related to satisfying biological requirements. Using a sacrificial bioink for a print material, the print volume is initially created and will subsequently be washed away, as shown in multiple approaches in literature illustrated in Fig. 46.8. The bioink provides space-filling volume and support that will be evacuated. Some groups refer to their sacrificial bioinks as fugitive inks to suggest its temporary role in printing in the scope of the final structure [103]. Therefore, a sacrificial bioink only needs to be nontoxic and will not introduce harmful by-products; however, no further biological features are necessary, such as cell adhesiveness or biodegradability [1]. Here, by nontoxic, we idealize success cases in which the by-products are also noncytotoxic. Still, sacrificial bioinks ideally match these specifications: high print fidelity, ease of removal, and the lack of toxicity (Fig. 46.8).

To enable ease of removal, an important material property is its gelation process: in other words, the conditions under which the printed material will wash away. Some examples of bioinks, such as Pluronic F127 or gelatin, have a thermally reversible gelation process. Therefore, although printing can occur at one temperature, the printed material can evacuate when another temperature is attained. The Lewis laboratory is one of the leaders using Pluronic F127 as a fugitive ink to create perfusable networks in tissue mimics. Pluronic F127, a poloxamer, is solid at 37°C but it can be liquefied when cooled to 4°C. They take advantage of this material property to flush out the fugitive bioink with cold cell media, leaving behind perfusable channels. The resulting print structures resemble thick vascularized tissue mimics with endothelialized lumens viable after 45 days of perfusion [12]. The same group applies this strategy to other tubular tissues; the technique was also applied to fabricating renal proximal tubules [86].

Other common sacrificial bioinks include agarose [67], alginate [57], and gelatin [66]. The Khademosseini laboratory applied sacrificial templating strategies with different materials. For agarose, the geometry was extruded to form a solid network at 4°C. Subsequently, the agarose fibers could be manually removed or lightly vacuumed because the material does not adhere to surrounding photocross-linked hydrogel [67]. With a different material, the Khademosseini cohort used sodium alginate to fabricate a sacrificial network. The gelation of alginate occurred ionically with calcium chloride and could be removed with EDTA treatment [57]. Others applied this strategy to 3DP other tissue mimics such as aortic valves and bone; however, those groups did not print with this bioink sacrificially

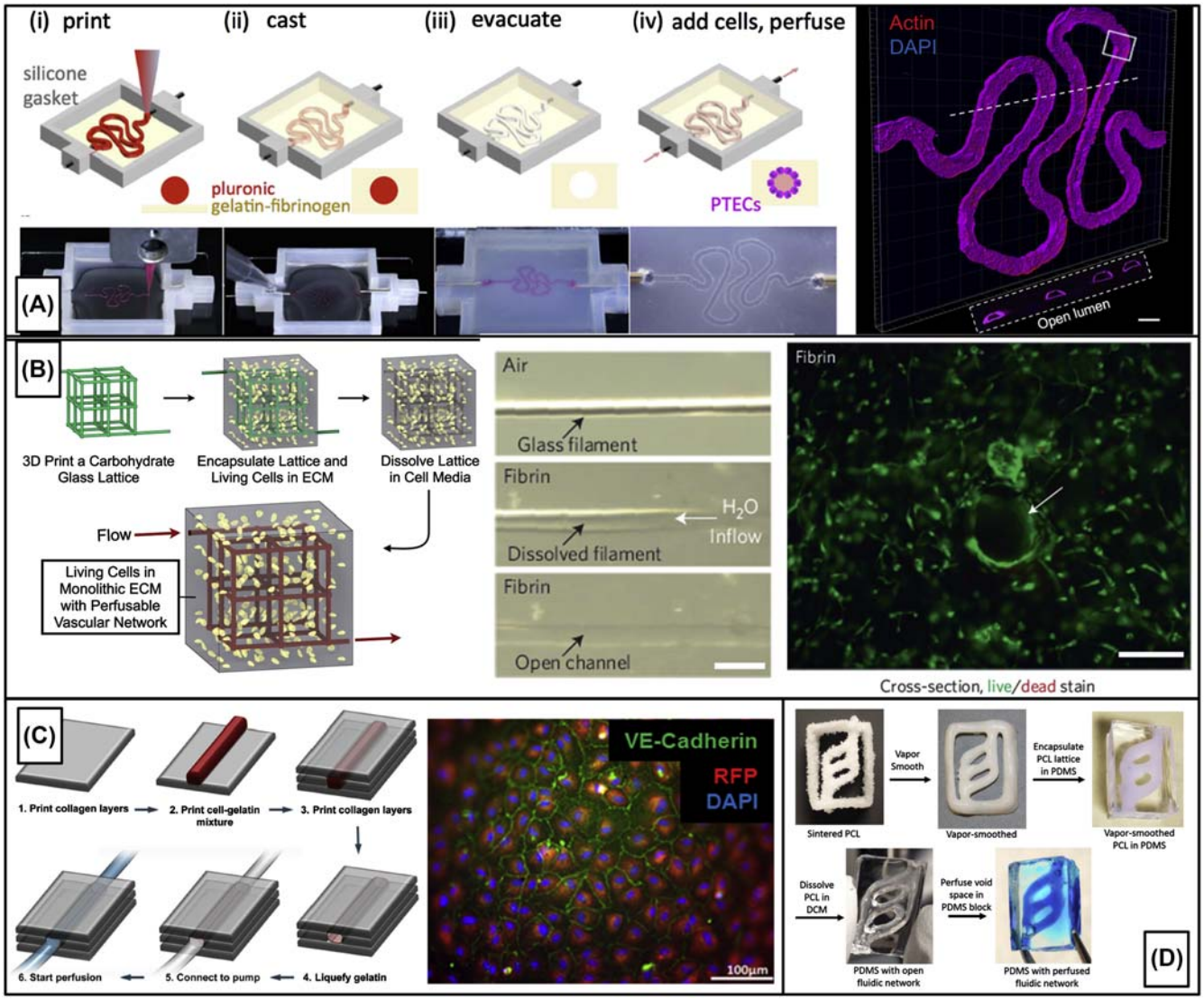

FIGURE 46.8 **Sacrificial templating strategies:** (A) The Lewis laboratory extrudes Pluronic F127 filaments in a renal proximal tubule geometry that is cast with a gelatin–fibrinogen gel. The extruded Pluronic can then be flushed out by cooling to 4°C to leave behind perfusable renal tubules that can be seeded with proximal tubule endothelial cells [86]. (B) Miller and colleagues similarly extrude a lattice structure with carbohydrate sugar glass that can be encapsulated with a number of different extracellular matrix (ECM) or ECM-like materials and later dissolved for a perfusable vasculature. Human umbilical vein endothelial cells are seeded in the casted gel and remain viable owing to the perfusion of cell media through the vascular architecture [11]. (C) Lee et al. print collagen layers with intermittent sacrificial and cell-laden gelatin filaments via inkjet. Gelatin's gelation process relies on thermal transition; therefore, it can be liquified to evacuate open geometries. When seeded with endothelial cells, junctional markers confirm their barrier function after long-term perfusion [66]. (D) Kinstlinger et al. demonstrate that selective laser sintering can also be implemented with a sacrificial templating strategy. Vascular geometries can be formed with poly(ε-caprolactone)(PCL) and casted over with polydimethylsiloxane (PDMS) (shown here) or other materials and leave behind open channels [29]. *3D*, three-dimensional; *DAPI*, 4′,6-diamidino-2-phenylindole; *PTECs*, proximal tubular epithelial cells; *RFP*, red fluorescent protein; *VE-cadherin*, vascular endothelial cadherin.

[58,104]. An additional naturally derived sacrificial bioink, gelatin, is thermally reversible, which proves useful for a sacrificial templating strategy. Lee and colleagues used inkjet printing to form vascularized tissue constructs dropwise and layer by layer with gelatin and collagen. Collagen layers were printed and polymerized at 4°C with a pH-altering cross-linking agent (sodium bicarbonate) so that the thermal responsive nature did not take precedence over the intended material evacuation. Within the collagen layers, gelatin was left uncross-linked and therefore was removed by liquefying the printed structure when the final print structure was raised to room temperature [66]. These natural sacrificial bioinks are all able to be easily evacuated, which fits one of the crucial characteristics of a sacrificial bioink.

So far, the sacrificial bioinks that have been discussed have poor mechanical strength potentially leading to complications in maintaining print fidelity after the print. Some researchers have addressed this complication by using an alternative sugar material that has high mechanical stiffness and is water soluble [11,105]. Miller and colleagues developed an approach to using carbohydrate sugar glass to fabricate complex vascular designs. This strategy circumvented issues related to poor mechanical strength. However, a similar evacuation approach is employed in which the printed perfusable network is evacuated by running cell media through the channels to provide a fluidic network. Although the carbohydrate glass lattices were printed at high temperatures (110°C, a temperature physiologically unviable), the finished print volume could be brought to physiological temperature with complete cell media for 10 min to dissolve the carbohydrate glass [11]. After evacuation, these open channels could be perfused with cell suspensions for long-term cell culture. However, the extrusion-based printing technique used to fabricate structures has limitations. One example of a limitation is that because the build volume relies on depositing material layer by layer, this technique is unable to create overhanging structures without support.

Some in the bioengineering community employ SLS as a different 3D bioprinting technique extending the list of bioinks while addressing the overhang limitation of extrusion-based approaches. SLS-based prints have been commonly used with materials such as PCL and PLA to produce bone-mimicking scaffolds, for example. However, emerging research for this printing strategy falls under the scope of sacrificial bioinks [106,107]. Limited in resolution only by the smallest powder size of the bioink and the laser used, SLS exhibits micron-sized fabricated structures with complicated overhangs with a sacrificial templating workflow [29]. Using PCL, a lattice structure can be selectively laser sintered and dissolved to leave behind a fluidic network. However, to provide perfusable channels, the printed construct requires dissolving in dichloromethane, which may or may not induce toxicity to future seeded cells [29]. More gentle evacuation for future cell encapsulation may be necessary to translate to a cytocompatible workflow. Although others use sacrificial templating strategies with plastics (i.e., acrylonitrile butadiene styrene) to fabricate intricate microfluidic systems and subsequently remove them with acetone, these removal approaches may not lend well to tissue incorporation [108].

Although there is a short list of sacrificial bioinks, these materials share one thing in common: the need to have multimaterial integration for a finalized structure. Inevitably, for the sacrificial ink to be evacuated, a requirement is an encapsulating material in which the flushed material can leave behind a space. The selection of the encapsulation material, the matrix, or matrix-mimicking bioink (see Extrusion-Based Printing section) is crucial to incorporating cells, or otherwise an interface with biological tissue might require cell-adhesive sites or degradation properties. Moreover, the gelation process of the encapsulation should ideally mismatch the sacrificial bioink so that the intended material is the one being sacrificed at the point of dissolving.

Supporting Bioinks and Supporting Baths

Supporting bioinks and supporting baths are used during 3DP to improve the mechanical properties and expand the geometric capacities of the bioprinted scaffold, as shown in Fig. 46.9. High viscosity is commonly a desired material property for supporting baths to provide structural integrity as the print is fabricated. This material property functions to hold the print in place so that the printed material alone (without the support bath) would be unable to be maintained (i.e., overhanging features or ultrathin features). Consistent with requirements for other bioinks, the material should not possess toxicity, to ensure biocompatibility.

The Feinberg laboratory takes an innovative approach to bioprinting by taking advantage of material properties to extrude bioprinted features within a hydrogel support bath. Their approach, coined freeform reversible embedding of suspended hydrogels, uses a gelatin slurry hydrogel bath embodying thermoreversible properties and Bingham viscosity to print soft, fragile constructs within the bath material. With the bath's Bingham plasticity, the material acts as a solid unless a yield shear stress is attained, in which case the material will flow as a viscous fluid locally to the applied shear. In this way, extruded cross-linked printed structures (i.e., fibrin, alginate, and collagen type I via enzymatic, ionic, and pH mechanisms- respectively) within the hydrogel bath will immediately be surrounded by the supporting viscous material- enabling improved complexity in fabrication without collapsing or deforming under the printed structure's own weight. In addition, the gelatin slurry possesses thermoreversible and biocompatible properties. The temporary support bath can be removed by raising the temperature from room temperature maintained during the printing process to a physiological range at 37°C (for gelatin specifically) to release the 3DP object supported within. Because the gelatin slurry is biocompatible, cells can be incorporated in the extruded bioink within the bath without concern regarding its effect on cell viability[23].

Similarly, others have achieved the bioprinting of intricate structures that otherwise cannot be achieved without support baths. Carbopol is another support bath material possessing desirable rheological properties in that the

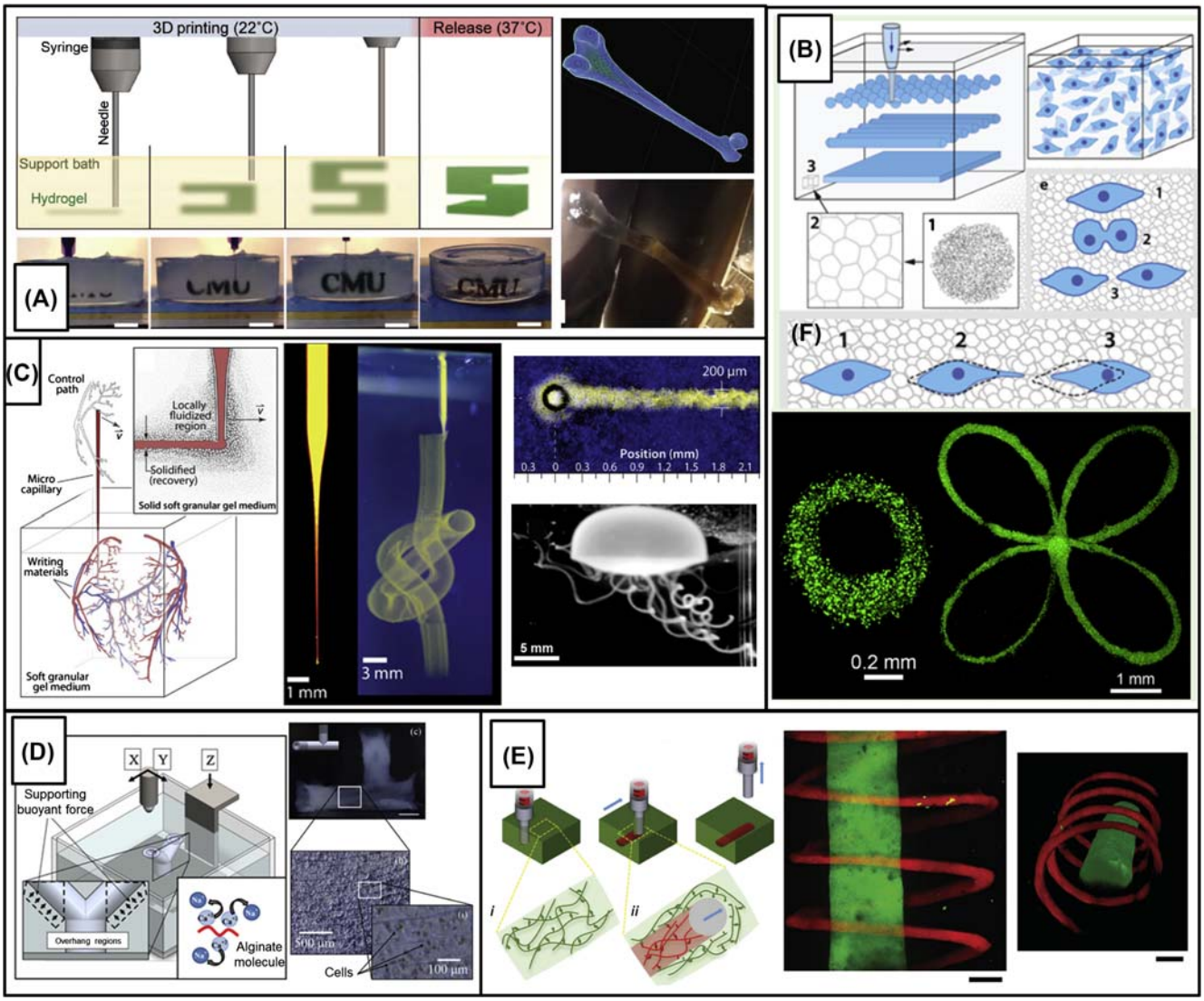

FIGURE 46.9 **Supportive baths used in three-dimensional (3D) bioprinting:** (A) The Feinberg laboratory developed the free-form reversible embedding of suspended hydrogels method to 3D printing (3DP) structures within a support bath. The bath is a Bingham plastic gelatin slurry that provides structural integrity to the print; an example is a human femur computed tomography scan that was downsized to create a printed version composed of alginate (scale = 1 cm, 4 mm) [23]. (B) Angelini's group demonstrates the capacity of cells to remain functional (viable, migrating, and proliferative) with their 3DP method [110] that expanded from their previous work in (C). In (C), a 3D object is printed within another gel support bath. This support bath, based on carbopol, fluidizes locally to the point at which shear is applied (i.e., the needle head extruding print material). The print can be released and form intricate structures such as the fine tentacles of a jellyfish [89]. (D) Christensen et al. use calcium chloride as a supporting and cross-linking bath for sodium alginate bifurcated objects with inkjet printing. This material supports cell encapsulation and allows for overhanging geometries [61]. (E) The Burdick laboratory uses photosensitive hyaluronic acid bioinks printed within a self-healing support hydrogel to form free-standing geometries, as shown by the spiral enveloping a channel [50].

transition from locally fluidized to solidified afforded by its Bingham plastic nature allows for the support of printed structures. Although the writing medium is more permanent (via photocross-linking or other means), the support bath can be dissolved with water [89,109]. The Angelini group has even expanded their carbopol support material to be cytocompatible for a range of cells (i.e., MSCs, human aortic endothelial cells) with enough elasticity to support cell division with the inclusion of cell media adjusted to pH 7.4 [112].

For those using inkjet printing for their bioprinted constructs, a common support bath system employs a $CaCl_2$ solution that can dually function as a cross-linking agent and a support material. In this way, the alginate inkjetted

deposition with or without cells is fabricated with the support bath instantly polymerizing the material to allow for complex structures with overhangs [61].

Yet another extrusion-based bioprinting approach, the Burdick laboratory developed a hydrogel bioink format that is directly written into a self-healing support hydrogel based on guest−host complexes. Their system is based on noncovalent and reversible bonds with the application of shear. By directly writing into this material, the bonds of the support bath are disrupted by the physical stimulus but are quickly reformed after the shear is removed. This enables the capacity of injectable hydrogels and extrusion 3DP. Their writing bioink based on HA was chemically modified for photocross-linking; more important, the support bath possesses shear thinning properties to provide structural support to print freestanding structures [50] (Fig. 46.9).

Support baths must interact with the finalized structure in such a way that the material itself must still provide a minimal baseline of compatibility. Certainly, some approaches have been using the support bath as a reservoir of cells or perhaps cross-linking agents that will ultimately become part of the printed construct; therefore, the level of cytocompatibility is going to increase correspondingly. Regarding toxicity, beyond the baseline of cell compatibility in cell-laden prints, future studies might want to consider long-term effects on cytotoxicity. If residual components of support baths are cytotoxic, a few days after the print is not long enough to prove long-term cell viability and functionality. Finally, in parallel to sacrificial inks, these materials must be easily removable to decouple from the finalized printed construct.

Current Translation of Three-Dimensional Bioprinting

3D bioprinting has opened the door for opportunities in directly translating to the clinic. 3DP provides us with the ability to print tissue analogs by controlling the delivery of living cells and matching the appropriate material in a defined and organized manner. The control is promising because it is beyond defining the exact location as well as the sufficient number of cells in a multimaterial environment. 3DP has applications to tissue-engineering scaffolds, constructing cell-based sensors, physiological screening for drug and toxicity, and modeling tissue disease and tumors [100]. Here we will broadly characterize translation applications using in vitro and in vivo examples.

In Vitro Applications

In general, lab-on-a-chip style models can represent tissue analogs well by incorporating the many fluidic networks of our body (i.e., vasculature, lymphatics). An example of lab-on-a-chip, miniaturized, functional tissue mimics was discussed in the sacrificial section from the Khademhosseini laboratory. In their work, they incorporated embedded vascular networks in their 3DP constructs [67]. Many others have also dedicated their research to incorporating vascular fluidic networks, because vascularization remains a critical challenge in tissue engineering [11,29,86,103]. As discussed, beyond a few hundred microns of the diffusion limit, cells will not remain viable. However, even with many research groups studying vascularization strategies, many challenges remain, such as which vascular geometries to print and how to reach truly multiscale vasculature. By miniaturizing human tissue functional elements, studies can more accurately predict drug toxicity over animal models. Khademhosseini and colleagues delved into more complex in vitro models by developing liver-on-a-chip constructs. By developing a liver model, one can assess drug toxicity more adequately because the liver has the most important role in drug metabolism. Their liver model was biofabricated as a perfusable bioreactor with a direct-write printer to create hepatic spheroid-laden hydrogel structures [65].

In addition to better drug screening, in vitro models fabricated with 3DP approaches can better represent tumor models to help researchers elucidate cancer mechanisms. Efforts are being made to represent tumors 3D in models, because the progression of tumor metastasis is significantly different from that of a 2D counterpart [111]. The West laboratory modeled tumor angiogenesis with layer-by-layer tunable PEG hydrogels [112]. 3DP can provide cancer researchers with control over specific 3D microenvironments influencing nutrient transport and fluid shear stresses. With this kind of physiological mimicry, researchers can identify mechanisms directing tumor metastasis.

A major challenge for 3DP is scale for tissue engineering translation: Printers often have difficulty achieving large, clinical-size organ analogs with the microdetail of the cellular organization necessary to be functional. The Atala laboratory reported an integrated tissue-organ printer that prints cell-laden hydrogels of desired mechanical stiffness with sacrificial hydrogels. Fabricated constructs presented include mandible bone, cartilage, and skeletal muscle. The printed anatomical shapes can be composed of multiple biomaterials and cells resulting in structures that in the future can be vascularized and included in more complex, solid organs [113]. Until then, these constructs may be difficult to incorporate in the clinic because encapsulated cells will quickly necrose without proper vascularization.

For in vitro 3DP, clinical translation is mostly limited to drug screening and disease modeling. These applications can provide important information regarding addressing drug toxicity and to develop drugs.

In Vivo Applications

The promise of 3DP to fabricate tissue parts for implantation in the body is taking shape in in vivo applications. Tissue engineering has the potential to facilitate tissue regeneration by replacing injured parts and encouraging regrowth with the appropriate healing environment (i.e., growth factors, vascularization, stem cells). With 3DP, a diversity of critical-sized defects can be addressed tailored to the needs of the individual. In this section, we discuss studies that use 3DP to fabricate scaffolds applied in vivo.

As mentioned previously, rapid vascularization is a limitation in applying tissue mimics in vivo, because cells will necrose soon after implantation without access to nutrients and oxygen. Several studies achieve rapid vascularization in vivo enabled by prevascularization strategies fabricated with 3DP techniques [114,115]. Using sacrificial templating with a sacrificial carbohydrate bioink, an open microvascular network can anastomose in line with the rat femoral artery with a surgical technique [11,114]. Similarly, others employ 3DP biodegradable scaffolds to anastomose built-in vasculature directly and surgically in an AngioChip to the host vasculature of the rat hind limb femoral vessels [115]. Patency is maintained through the fabricated vessels within the scaffold. These studies demonstrate the potential of prevascularizing tissue analogues with 3DP for rapid vascularization in vivo.

Other tissues have been fabricated and implanted in vivo, such as bone. Bone scaffolds 3DP with MSCs, bone morphogenetic protein growth factor, and PCL as the bioink have been implanted in critical-sized bone defects of sheep. After 3 and 12 months, bone healing progresses with signs of vascularization ingrowth, mineralization, and appropriate mechanical stiffness [76]. By considering important aspects of biocompatibility and degradability, many of the bioink principles described earlier here have resulted in the success of 3DP tissue mimics for in vivo implantation.

The opportunities to apply 3DP to in vivo applications are still being explored. Integration with the host tissue is of utmost importance for all in vivo studies. For researchers, great care is taken to select the most appropriate bioink during fabrication to allow the best possibility for successful integration. Future work is necessary to ensure the functionality of the intended tissue analog taking into consideration the role of the parenchyma.

CONCLUSION AND FUTURE DIRECTIONS

This chapter introduces current 3DP techniques and describes the materials used to enable bioengineering applications. For bioprinting, the materials, or bioinks, used for fabrication must be biocompatible as well as printable. Bioinks are printing materials that will interface with biological components or are part of the fabrication process of a construct that will come into biological contact. We describe three separate categories for bioinks: (1) matrix or matrix-mimicking, (2) sacrificial, and (3) supporting.

Although there has been great progress in synthesizing biocompatible and printable materials, future work is needed to address the greatest limitations in bioengineering. For one, vascularization must be rapidly integrated. Many of the challenges for vascularization strategies involve scale to realize multiscale hierarchical vessels. Reaching capillary-sized vessels requires microscale resolution in printing. However, many printing techniques that can even reach these small levels are not able to print rapidly. Another limitation in current studies is the use of physiologically relevant cell types. Fortunately, induced pluripotent stem cells hold much promise in alleviating this limitation. Materials available as bioinks are limited. Generally, the bioinks used are a compromise between structural strength and biocompatibility. Therefore, current and future work follows a multimaterial approach to achieve the desired properties.

The advent of 3DP applied to bioengineering and tissue engineering has pushed researchers to create complex biological structures with incorporated fluidic networks with multiple cells and materials for the most physiologically mimicking environment. This is an exciting time for the field to realize approaches that have a clinical impact. From rapidly reiterating studies for drug screening to achieving high spatial resolution to fabricate tissue analogs, 3DP holds much promise for therapeutic translation. The choice of bioinks will continue to have a fundamental role in determining ultimate biocompatibility.

List of Acronyms and Abbreviations

2D Two-dimensional
3D Three-dimensional
3DP Three-dimensional printing
$CaCl_2$ Calcium chloride
dECM Decellularized extracellular matrix
ECM Extracellular matrix
EDTA Ethylenediaminetetraacetic acid
gelMA Gelatin methacrylate
HA Hyaluronic acid
hCMPC Human cardiac-derived cardiomyocyte progenitor cell
MSC Mesenchymal stem cells
MTU Muscle–tendon unit
PCL Poly(ε-caprolactone)
PDMS Polydimethylsiloxane
PEG Poly(ethylene glycol)
PEGDA Poly(ethylene glycol) diacrylate
PEGX Poly(ethylene glycol) with reactive ends
PGA Poly(glycolic acid)
PLA Poly(L-lactic acid)
PLGA Poly(D,L-lactic-*co*-glycolic acid)
PPF Poly(propylene fumarate)
SLS Selective laser sintering
UV Ultraviolet

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CHAPTER

47

Three-Dimensional Tissue and Organ Printing in Regenerative Medicine

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INTRODUCTION

Attempts have been made in tissue engineering to develop biological substitutes to address the high shortage of tissues and organs for transplantation. Although tissue engineering has progressed rapidly over the past 2 decades, conventional fabrication methods are limited in their ability to create clinically applicable tissue constructs with well-interconnected pores, complex architectures, patient-specific geometries, and heterogeneous material distributions. Over the past few years, three-dimensional (3D) bioprinting strategy has been applied to overcome these limitations. It enables the fabrication of tissue constructs with unique spatial control over the deposition of cells, biomaterials, and bioactive molecules such as growth factors, cytokines, peptides, and small molecules, resulting in higher regenerative capability after implantation [1,2]. 3D printing technology was developed in the 1980s and included various approaches to creating objects from a computer-generated file [3]. This technology quickly became a powerful tool in tissue engineering and biomedical research [4]. In structure-based bioprinting, bioinert or bioactive materials such as metals, ceramics, and polymers are used to develop a tissue structure followed by precisely depositing cells and bioactive molecules onto it [5,6]. In cell-based bioprinting, a high density of cells is patterned spatially with a prescribed organization in a layer-by-layer fashion, forming tissue constructs [5].

Thus, additive biomanufacturing or 3D bioprinting allows the creation of tissue-specific architectures with precise geometries that have been limited using conventional fabrication methods. Landers et al. first introduced 3D bioprinting as an extrusion-based method to dispense cells continuously within a hydrogel material (bioink) from a dispensing head to a stage-based patterns predesigned through computer-aided design/computer-aided manufacturing (CAD/CAM) tools [7,8]. Various types of 3D bioprinting methodologies are available to meet specific requirements in tissue engineering applications.

BIOPRINTING STRATEGY: FROM MEDICAL IMAGE TO PRINTED TISSUE

Bioprinting aims to achieve reproducible, complex tissue structures that are well-vascularized and suitable for future clinical use. Human tissues and organs have arbitrary 3D shapes composed of multiple cell types and an extracellular matrix (ECM) with a functional organization. CAD/CAM processes are important technologies necessary for future clinical applications of 3D bioprinting because these processes provide an automated way to replicate the 3D shape of a targeted tissue structure. In general, the process starts by scanning the patient to produce 3D volumetric information about a target object using medical imaging modalities such as computed tomography

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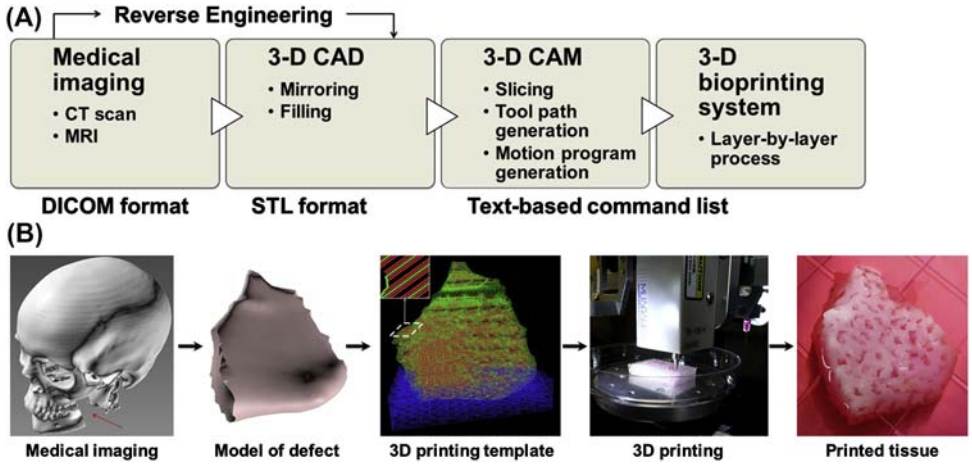

FIGURE 47.1 Three-dimensional (3-D) bioprinting strategy from medical imaging to printed tissue construct: (A) schematic diagram and (B) example of computer-aided design (CAM)/computer-aided manufacturing process for automated printing of 3D shape imitating target tissue or organ. A 3D CAD model developed from medical image data generates a visualized motion program, which includes instructions for XYZ stage movements and actuating pneumatic pressure to achieve 3D bioprinting. *CT*, computed tomography; *DICOM*, Digital Imaging and Communications in Medicine; *MRI*, magnetic resonance imaging; *STL*, stereolithography.

(CT) and magnetic resonance imaging. These imaging tools acquire information from cross-sectional slices of the body and the data are stored in the Digital Imaging and Communications in Medicine format, which is a standard format for digital imaging in medicine. This information is transformed into a 3D CAD model by reverse engineering. This process starts by interpolating points within and between image slices to improve the resolution and generate voxels from the measured data. This CAD model is created by extracting localized volumetric data from a targeted tissue structure to generate a surface model. In this step, sophisticated reconstruction of the CAD model is required for bioprinting owing to the complexity of the tissue or organ. A motion program, which is instructional computer codes for the printer to follow designed paths, is generated with a CAM system. This CAM process is divided into three steps: slicing, the tool path, and the generation of the motion program. Slicing obtains information about sliced 2D shapes of an object for the layer-by-layer process. Then, tool path generation creates a path for the tool to follow to fill the cross-sectional space of each layer. The printed tissue-specific structure has the proper inner architecture constructed with multiple cellular components for efficient tissue regeneration. Therefore, a well-organized strategy for tool path generation is required to have high efficient tissue regeneration and is an important process for 3D bioprinting. Fig. 47.1 shows the 3D bioprinting strategy from the medical image to the printed tissue constructs developed by CAD/CAM process and automated printing of a 3D shape imitating a target tissue or organ.

BIOPRINTING MECHANISMS

Researchers have developed a variety of printing mechanisms, all of which aim to accomplish the same goal: printing 3D human tissue or organ structures for reconstruction. The effectiveness of each printing mechanism relies heavily on the choice of biomaterials and the targeted applications. Bioprinters consist of three main components: a three-axis stage, printing cartridges, and the dispenser. Stage controllers are used to move the printer head in the X, Y, and Z directions. Printing cartridges, usually in the form of a syringe, store either the polymeric components of the scaffold or the cellular/hydrogel components. They contain the nozzle, which determines the amount of material dispensed at set printing parameters. The dispenser is the final component; it varies among printing methods, but it is the mechanism that deposits materials (Fig. 47.2).

Jetting-Based Printing

Jetting-based printing, or "inkjet" printing, is the most commonly used printing mechanism for both nonbiological and biological objects [9]. Similar to an inkjet printer used to apply ink to paper, jetting-based bioprinters dispense a controlled volume of liquid to a predefined location through noncontact deposition (Fig. 47.2A). The "ink" in this instance is usually a hydrogel that may or may not contain cells and can be dispensed in

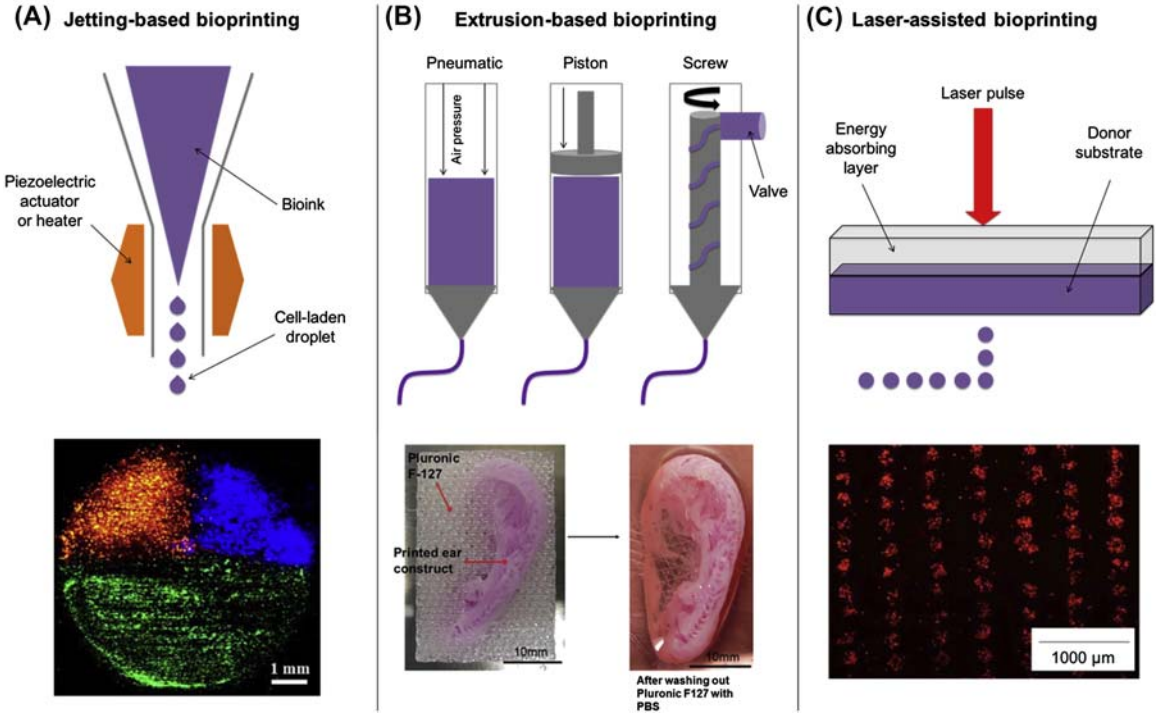

FIGURE 47.2 (A) Microscopic view of a three-dimensional multicell "pie" construct printed by a jetting-based mechanism. Individual cells were printed in predetermined locations and then located by microscopy at those locations after printing [124]. (B) Auricular implant was printed through extrusion-based mechanisms with cell-laden hydrogel, poly(ε-caprolactone), and Pluronic F-12 [47]. (C) Human umbilical vein endothelial cells were printed precisely in straight lines through laser-assisted bioprinting to study cell migration rates [125]. *PBS*, phosphate-buffered saline.

volumes between 10 and 150 pL, depending on the dispensing modules used [10]. The two main dispensing methods for jetting-based printing are thermal and piezoelectric inkjets. Thermal inkjets use an electric heater that generates small bubbles in the printhead. These bubbles collapse to create pressure pulses that force droplets of liquid out of the nozzle. Although thermal inkjets use heat at around 200−300°C, the duration of heating is typically around 2 μs, which studies have found to result in only a 4−10°C rise in hydrogel temperature [9]. Piezoelectric inkjet printers use a polycrystalline piezoelectric ceramic to create the pressure pulse that ejects the droplet [10]. The volume of liquid dispensed depends on the temperature gradient, the frequency of the pressure pulse, and the ink viscosity. To determine where the material will be dispensed, the bioprinter can control the X and Y positions of the nozzle heads to a scale of micrometers, which allows for precise, high-resolution printing.

A major drawback of jetting-based printing is the amount of shear stress induced on the cells as they are forced through the nozzle. Shear stress creates a huge risk to damage cell membranes and causes cell lysis [10]. Another drawback of this printing is that to dispense materials, it has to be in a liquid form, so to form a solid structure, the liquid has to be cross-linked during or very soon after printing. This causes a significant reduction in printing speed, because a time for cross-linking must be determined. It is also considered that high cell concentrations (greater than 10 million cells/mL) often result in nozzle clogging and some instances may even alter the properties of the hydrogel so much that it can no longer be cross-linked [11,12].

Despite these drawbacks, jetting-based printing offers many advantages over laser-assisted and extrusion-based printing methods. Commercial inkjet printers are readily available, and because of this, many laboratories can modify the printers so that they can experiment with bioprinting at a low cost. The printers also offer high printing resolution, which allows concentration gradients of cells, biomaterials, or bioactive molecules to be introduced throughout the structure by altering drop sizes and densities [9]. The droplets can be arranged into patterns such as lines 50 μm wide or single droplet patterns containing one or two cells [13]. The droplet size can be controlled electronically and can range from less than 1 pL to more than >300 pL in volume with deposition rates from 1 to 10,000 droplets per second [14].

Extrusion-Based Printing

Unlike jetting-based printing, extrusion-based printing is an additive manufacturing mechanism that relies on fused deposition modeling and requires direct contact with a surface to print (Fig. 47.2B). These printers mainly include a temperature-controllable dispensing system and a stage, with one or both capable of movement in the X, Y, and Z axes. Some 3D extrusion printers have multiple dispensers that allow them to switch printing material without going through the process of retooling [15]. Extrusion-based printing methods offer improved printing resolution, speed, and spatial control. These printers also differ from inkjet printers in the manner in which they deposit material, because they dispense in a continuous string as opposed to individual droplets. The printer extrudes a 2D pattern designed by CAD/CAM software, and then each layer serves as the foundation for the next layer above it. Three systems can be used to extrude the material: pneumatic, piston, or screw-based dispensing systems [9]. Pneumatic dispensing systems can deposit high viscous materials, but the drawback is that there is a delay in deposition owing to the time it takes to compress the gas in the cartridge. The ultimate force of pneumatic systems is limited only by the air-pressure capabilities of the system. Mechanical systems such as a piston and screw-based dispensers provide more spatial control at the cost of reduced maximum force capabilities [9].

Extrusion-based printing methods have the widest range of biomaterials that can be printed; they vary from hydrogels to polymers and cell aggregates. Materials with viscosities ranging from 30 mPa/s to greater than 6×10^7 mPa/s have been successfully printed with extrusion-based printers, with high viscous materials acting as structural support and low viscous materials providing an adequate environment for cell printing [16]. This printing method is also able to print a high density of cells; however, cell viability may be decreased with smaller nozzle sizes and a high pressure level. The main challenges to extrusion-based printing mechanisms are the low printing resolution and low printing speed: 5- to 200-μm pattern resolution at linear speeds of 10−50 μm/s [17].

Laser-Assisted Printing

Laser-assisted bioprinters operate by focusing a laser pulse toward an absorbing layer, typically gold or titanium, to generate high-pressure bubbles that propel cell-containing bioinks toward a collector substrate [9]. A standard laser-assisted printing system usually consists of a pulsed laser beam, a focusing system, a "ribbon" that has a transport support made from a laser energy−absorbing layer, a layer of hydrogel or cellular material, and a substrate-receiving layer (Fig. 47.2). Laser-assisted printing method has successfully transferred peptides, DNA, and cells [18]. The main advantage of this method is that it is nozzle-free; therefore, it avoids nozzle clogging issues found in other bioprinting systems. Laser-assisted methods can deposit materials with viscosities ranging from 1 to 300 mPa/s and cell densities close to 10^8 cells/mL, with resolutions close to a single cell per drop without significant effects on cell viability or function. In addition, many factors affect the printing resolution. Some of these factors include the energy delivered per unit area owing to the laser, the surface tension, substrate wettability, the gap between ribbon and substrate, and the thickness and viscosity of the biological layer [9].

There are also a lot of disadvantages associated with the laser-assisted printing system. One drawback is that each ribbon must be prepared following a time-consuming process that may become overwhelming if multiple cells or hydrogels must be codeposited [9]. It can also be difficult to target and position the cells accurately because of the nature of the ribbon cell coating. Metallic residues may be present in the final construct owing to vaporization of the laser-absorbing layer, although there have been methods to reduce this contamination, including using nonmetallic absorbing layers and altering the printing process so that an adsorbing layer is not needed [19].

Hybrid and Other Mechanisms

Hybrid fabrication is a combination of technologies that are practiced individually. Despite rapid growth over 2 decades, current 3D bioprinting techniques and systems fall short in integrating rigid and soft multifunctional components. In some instances, the advantages of one technology might not be sufficient to meet the requirements of creating targeted functional tissue constructs. Merging technologies may overcome this limitation and improve the bioprinting process. The combination of technologies can be at the cellular, bioink, or bioprinter level. Tan et al. introduced a 3D bioprinting strategy by integrating conventional scaffold-based fabrication and the cell-based bioprinting approach [20]. They explored the use of hydrogel-encapsulated, cell-laden microspheres as the bioink for 3D bioprinting. The hydrogel lubricates and glues the microspheres during printing and fuses them together

after printing upon gelation. Kucukgul et al. developed computational algorithms to generate support structures for cylindrical cell aggregates to print fully cell-based vascular structures [21]. Xu et al. proposed a combination of electrospinning and inkjet bioprinting techniques to develop layered cartilage constructs [22]. Combining the two principles of electrospinning and bioprinting resulted in the fabrication of a cartilage construct with appropriate cell function and mechanical properties. Shanjani et al. developed a new hybrid 3D bioprinting technology called Hybprinter by integrating soft and rigid components [23]. This technique employs a digital light processing–based stereolithography component and molten material extrusion techniques. Poly(ethylene glycol) diacrylate (PEGDA) was used as soft hydrogel and poly(ε-caprolactone) (PCL) as the scaffold's structural support. Mendoza-Buenrostro et al. reported a hybrid bioprinting technique for the fabrication of scaffolds with topography scales ranging from nanometers to millimeters [24]. Scaffolds were produced by extrusion-printing PCL embedded with nanofibers.

BIOMATERIALS FOR BIOPRINTING: BIOINKS

Printability

When used in bioprinting, hydrogels that can encapsulate and deliver cells and bioactive molecules through printing mechanisms are referred to as bioinks. In extrusion-based bioprinting, bioinks should have strong shear thinning behavior, which means that viscosity decreases under shear strain [25]. They should also have low surface tension so that extrusion is possible without the formation of droplets at the needle tip. In addition, bioinks should have proper viscoelastic properties that allow them to be stable enough to be printed in multiple layers before cross-linking. Traditional hydrogel precursors are low-viscous solutions that are cross-linked either during or after the printing process. Cross-linking before printing increases the shear stress on the cells, resulting in cellular damage and nozzle clogging. Cross-linking after printing affects the resolution because of the spread of bioink in the time between extrusion and cross-linking and can lead to incomplete cross-linking deep in large, multilayered constructs [9]. Fig. 47.3 shows a schematic diagram of variables, including printability, in 3D bioprinting.

There are structural properties that must be satisfied so that the printed construct maintains shape, accuracy, and integrity after printing is finished. The printed construct should maintain its predesigned structure, which includes shape, resolution, orientation, and mechanical properties. Because bioprinting aims to build a 3D tissue construct with cells, cell viability after printing is one of the main criteria for the printability of bioinks. For the cells to survive, a biologically favorable microenvironment is required so that the cells can be well-preserved not only during the printing process but also afterward in culture. In hydrogel-based bioinks, cell density, diffusion coefficient, temperature, and humidity can significantly affect the printability of the bioinks [26–28].

Hydrogel-Based Bioinks for Cell Printing

Hydrogels are naturally derived or synthetically produced. Hydrogel networks consist of polymer chains or peptide chains. They are highly moist and ideal for absorbing high levels of nutrients and oxygen, enabling cells to survive within the construct and diffuse waste. Therefore, hydrogels have been printed by a variety of mechanisms to deliver cells in the printed constructs. In the extrusion-based printing, the required properties of hydrogel-based bioinks during the printing process are (1) high viscosity to provide homogeneous cell suspension and initial structural integrity, (2) strong shear-thinning behavior to minimize cell damage, and (3) rapid gelation mechanism to stabilize a 3D-printed structure. If the gelation time is too long, the spatial resolution is lost and the layer cannot be printed correctly. To control the gelation time, the cross-linking mechanisms can be manipulated by chemically modifying the material, introducing a cross-linking agent or changing the polymer content [29].

Synthetic Hydrogels

Synthetic hydrogels used as bioinks have low cytotoxicity, controlled biodegradability, and good mechanical properties; however, most synthetic hydrogels have low biological properties that interact minimally with cells. Pluronic F127, a thermosensitive hydrogel, undergoes a phase transition at room temperature and becomes a viscous substance [30]. When the concentration of Pluronic F127 is 25% w/v or more, it can be dispensed with high printability. Although high-resolution of the printed structures can be achieved using Pluronic F127, the structure can

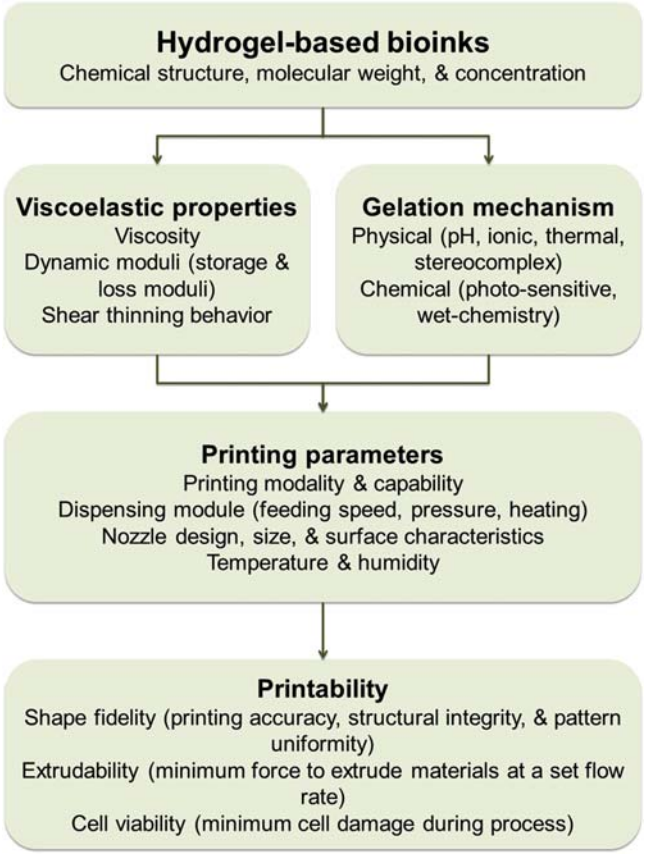

FIGURE 47.3 Schematic diagram of variables critical to three-dimensional bioprinting strategy. The hydrogel-based bioinks determine the viscosity, gelation mechanism, and printing parameters, and eventually the bioprinted tissue structures.

easily be collapsed in the culture condition. To improve its mechanical stability, Pluronic F127 has commonly been used chemically modified as a photocross-linkable hydrogel [31]. Poly(ethylene glycol) (PEG)-based hydrogels can be chemically modified to control their biological and biomechanical properties. Therefore, PEGs have been used as bioink materials in 3D bioprinting by introducing various functional motifs attached to the terminal end of PEG [32] or combining them with other hydrogels [33,34].

Naturally Derived Hydrogels

Natural hydrogels are classified into mainly proteins and polysaccharides. Most of them are present in the body, so they show high biological properties and do not cause a severe immune response. Collagen (type I) is the most abundant component of ECMs; it contains cell-guiding chemical cues such as the cell adhesion peptide sequence arginine-glycine-aspartic acid. Under the appropriate temperature and pH, a pure collagen solution physically forms a gel with properties dependent on the solution concentration. However, collagen itself has rarely been used as a bioink material owing to low viscosity and poor mechanical stability. To overcome these limitations, collagen has been mixed with various other hydrogels such as agarose, chitosan, fibrin, and hyaluronic acid (HA) [22,35–37]. An approach to printing skin cell-containing collagen solution from separate nozzles using an inkjet microvalve dispensing method was reported [38]. In the printing process, the collagen solution remained acidic and cooled. Multiple layers of cell-laden collagen bioink were printed and then treated with aerosolized sodium bicarbonate ($NaHCO_3$) to buffer the pH toward neutral for gelation. The results showed high cell viability at 1 day after printing for keratinocytes and fibroblasts, which indicated the survival of cells and spatial control of the printing approach, which is needed to offer a functional skin replacement.

Gelatin is a substance in which water-insoluble collagen is made soluble by high temperature or acid–base treatment. It forms a thermoreversible hydrogel. Moreover, chemical modification of gelatin with unsaturated methyl methacrylate results in gelatin methacrylate (GelMA), which can form covalently cross-linked hydrogels

under UV exposure [39]. Photocross-linkable GelMA is the most popular bioink material for cell bioprinting because of high printability and tunable mechanical properties. GelMa was used to print a complex architecture that contained various cell types and vasculature [40]. A cell-laden GelMa and sacrificial Pluronic F127 were dispensed and embedded within the GelMa block in a predetermined 3D structure. Afterward, the printed structure was cross-linked by UV exposure. The sacrificial Pluronic F127 was removed at 4°C by phase transition to create open microchannels within the GelMa block. Finally, a suspension of human umbilical vein endothelial cells (HUVECs) was seeded into the open microchannels. This approach allowed for the viable deposition of cells in a 3D structure with a microvessel-like structure that was covered by endothelial cells (ECs) to provide nutrients to surrounding cells.

HA is the most abundant of the glycosaminoglycan (GAG) family; it has the repeating structure of glucuronic acid and *N*-acetyl-glucosamine disaccharide. HA has a high molecular weight and a large amount of branching, which allows for intermolecular hydrogen bonding and high viscosity. Like other polysaccharides, HA can support cell survival but has low cell-binding motifs. Moreover, HA itself has low structural integrity and shape fidelity after printing; therefore, HA has been chemically modified for cross-linking or mixed with other hydrogels [41].

Fibrinogen, which is a glycoprotein, is reacted with thrombin to convert into fibrin network self-assemblies [42]. Fibrin has many cell-binding motifs that enable cell attachment and vulnerability to proteases for remodeling. Fibrin-based bioink has been used by laser-induced forward transfer–based printing for a 3D multicellular array [43]. To improve the gel stability, HA was added to a fibrinogen solution to print the arrays. Endothelial colony-forming cells (ECFCs) were printed along with adipose-derived stem cells (ASCs) in a 3D array to observe cell-cell interactions. These droplet arrays were printed onto a layer of fibrinogen–HA that was spray-treated with a thrombin–calcium chloride solution to induce gelation. The cell-laden droplets were converted into fibrin–HA as they encountered the treated substrate with a residual thrombin solution. Results showed that ASCs initially migrated toward ECFCs with no evidence of ECFC sprouting or migrating. Once ASCs contacted the ECFC aggregates, an explosion of ECFC network sprouts began to extend from the initial droplet position and remained stable networks for several weeks.

Alginate is a naturally derived anionic polysaccharide that exhibits gelation in the presence of bivalent ions such as Ca^{2+} [44]. Alginate hydrogel has served as a cell delivery material for many tissue engineering and drug delivery applications owing to its ease of preparation and relatively good cell compatibility; however, a major drawback is the lack of mammalian enzymatic degradation, which limits tissue remodeling when implanted. Because the solubility of alginate is low, it is often mixed with other materials to increase the printing resolution as a bioink. For example, alginate solution mixed with cellulose nanofibers improved shear fluidization and viscosity, resulting in high printing resolution. In addition, alginate was cross-linked with the divalent cation Ca^{2+} to stability the printed construct.

Tissue-specific, ECM-based bioinks have been developed and tested. Technically, ECMs obtained from various decellularized tissues are pulverized and solubilized as bioinks [45]. The approaches combined with tissue-specific ECM-based bioinks and tissue-specific cell types have been shown to be valuable for recapitulating anticipated tissue features [45,46]. However, the ECM solution inherently has a low viscosity and exhibits low shape fidelity and structural stability. Thus, various attempts have been made to enhance their chemical and physical properties in 3D bioprinting.

Biodegradable Synthetic Polymers for Structural Integrity

Synthetic polymers have some advantages for applications in tissue engineering and 3D bioprinting. These polymers can be synthesized with reproducible quality and fabricated into various structures with predetermined bulk and surface properties. Additional advantages include the ability to tailor their biomechanical properties and biodegradation kinetics for various biomedical applications. In bioprinting, synthetic polymers such as PCL, poly(lactide-*co*-glycolide) (PLGA), and poly(lactic acid) can provide mechanical strength, thereby overcoming limitations of the hydrogel-based constructs regarding size, shape, and structural integrity [47]. For the extrusion method, melted polymers or polymer solutions with proper viscosity are needed. Therefore, PCL is the most commonly available polymer for extrusion-based bioprinting because of its low melting point of 60°C and high printability. Many research groups are exploring the synthesis of polymeric biomaterials for 3D bioprinting.

Scaffold-Free Cell Printing

3D tissue structure can be printed using multicellular aggregates without supporting materials [48]. This method enables cell aggregates to be dispensed from a capillary to form a 3D structure. Norotte et al. reported a scaffold-free cell printing method using multicellular spheroids consisting of smooth muscle cells (SMCs) and fibroblasts and agarose hydrogel for temporary support [49]. After printing, the printed cell aggregates were fused and formed a small-diameter vessel-like tube ranging from 0.9 to 2.5 mm in diameter. Li et al. fabricated vertical channels without using a support material by taking advantage of the gelation behavior of gelatin combined with cross-linking alginate or fibrinogen [50]. Itoh et al. proposed a method to fabricate a scaffold-free vascular tube using cell aggregates [51]. Predesigned 3D tubular structures were constructed with cell aggregates consisting of ECs, SMCs, and fibroblasts. The multicellular aggregates self-organized and fused into a tubular structure that was perfused with a bioreactor. To avoid droplets from forming at the end of the nozzle owing to surface tension, Ozler et al. developed a quantitative model to predict the success of cell aggregate extrusion [48]. This approach can be repeated for different cell types after obtaining their respective rheological properties. In addition, cell viability can depend on the compression ratio applied during printing. Thus, it is necessary first to investigate the impact of compression on their survival rate and cellular functions.

THREE-DIMENSIONAL BIOPRINTING IN REGENERATIVE MEDICINE APPLICATIONS

Three-Dimensional Bioprinted Vascular Structures

A major limitation of bioengineered tissue constructs is the lack of proper vascularization into the implanted constructs. Fully vascularized tissue constructs are required to attain long-term cell survival and tissue functions. 3D tissue constructs packed with metabolically active cells can rapidly form necrotic cores in the absence of a vascular network [52]. The transport of nutrients and other physiologically relevant molecules toward and away from a large tissue construct is still limited [53,54]. When a 3D tissue construct with cells is implanted, efficient mass transfer requires an intact microvasculature to maintain the metabolic functions of cells deep inside the construct. Indeed, the ingrowth of a microvascular system into the implanted bioengineered tissue constructs in a timely manner is the key to success in clinical use [9]. Hence, many attempts have employed 3D bioprinting as a promising technology to fabricate vascularized tissue constructs (Fig. 47.4) [40,49,55].

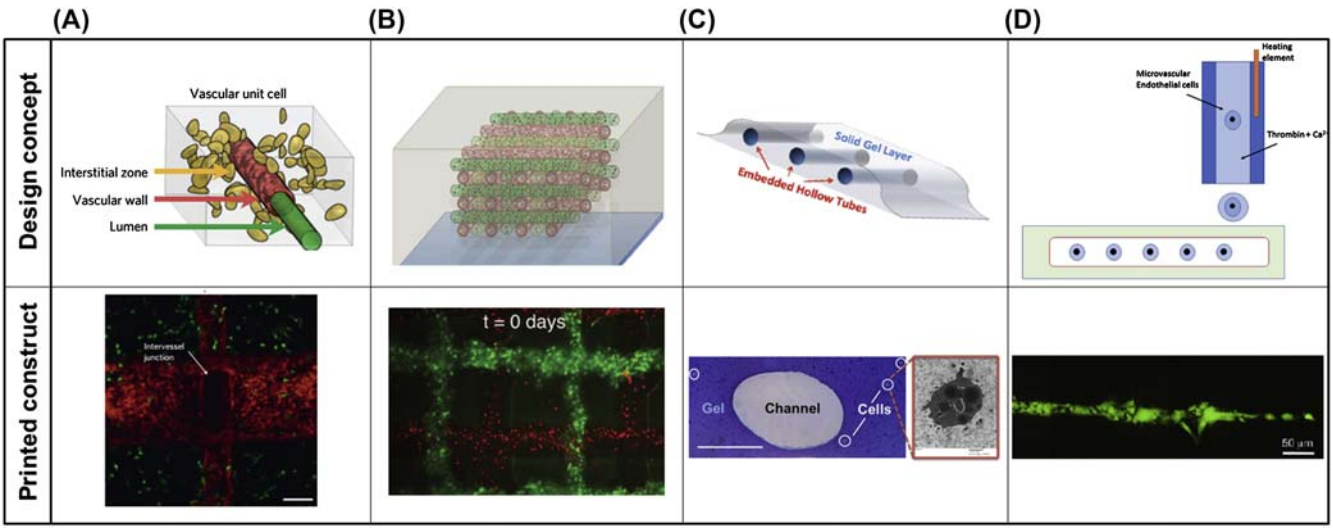

FIGURE 47.4 Examples of the three-dimensional bioprinted vasculature. (A) Vascular unit printed by Miller et al. [56] The construct shows three compartments consisting of the vascular lumen, the endothelial cell (EC) lining, and the matrix-encapsulated cells. Endothelialized channel walls and the intervessel junction surrounded by human fibroblasts. (B) Engineered tissue construct developed by Kolesky et al. [40] showing channels lined with human umbilical vein endothelial cells (HUVEC) (*red*) and human neonatal dermal fibroblast–laden gelatin methacrylate (*green*). (C) Hydrogel construct with embedded, connected hollow channels reported by Attalla et al. [57] and cross-section of hollow channel embedded within HUVEC-laden hydrogel. (D) Deposition of ECs and fibrin channel scaffold using thermal inkjet printer described by Cui and Boland [55]. Fluorescent image shows the printed microvasculature after 21 days.

One of the earliest and simplest approaches to fabricating a vascularized tissue construct is to use a sacrificial component that acts as a structural role during printing and is later removed to create a hollow tunnel. Miller et al. reported a vascular casting approach with carbohydrate glass as the sacrificial template; however, vascular networks developed by carbohydrate glass are limited in size and culture duration because of the practical difficulty of direct perfusion [56]. Kolesky et al. attempted to overcome these limitations by fabricating 3D constructs complete with a vasculature including multiple cell types and ECM proteins [40]. Attalla et al. modified a printhead with a microfluidic unit and generated instantly perfusable vascular networks integrated within a cell-seeded hydrogel [57]. The vascular structures were made from alginate using a coaxial nozzle. Cui and Boland reported an inkjet-based printing method with a precise droplet volume of 130 pL to fabricate microchannels [55]. Human microvascular ECs combined with the printing of biomaterials were found to align themselves inside the microchannels and form a confluent microvascular lining. Although bioprinting technologies have evolved to a level of creating complex vascularized constructs with multiple cell types and ECM proteins, reconnection of the vascular structures to the host circulatory system remains challenging. Lee et al. developed a novel bioprinting approach to creating a network from capillaries to large perfused vascular channels [58]. This was achieved by angiogenic sprouting from the large vessel through a natural maturation process. However, these printed constructs are often thin or hollow structures, so they are nourished by diffusion from the host vasculature but not by anastomosing [59]. When the diffusion limit needed by engineered tissues exceeds 150−200 μm, a precise vascular network must be embedded into fabricated constructs, a feat that has not yet been accomplished [60,61]. Efforts to simplify the complex fabrication methods and find new technologies for bioprinting vascular structures are in great need by tissue engineering as a whole.

In Vitro Tissue Models

Drug discovery is an inefficient process with a high failure rate and an extreme financial burden. Animal studies do not always indicate the results in human trials, and the regulatory environment is becoming stricter as time progresses. In addition, from a moral standpoint, attempts should be made to reduce the number of animal studies conducted. In vitro studies, mostly 2D cell culture methods are also severely limited. Drug response, gene expression, migration, morphology, and viability have all been shown to differ between 2D and 3D environments. Much emphasis has been placed on creating in vitro 3D tissue models to overcome these limitations. Typically, this is done by suspending cells or organoids (or cell aggregates) in a 3D culture within a singular or entire array of microfluidic devices. 3D in vitro assay systems have advanced immensely to a level in which living constructs can closely mimic the native tissue environment in a high-throughput platform. Although several fabrication techniques have been used to develop these models, 3D bioprinting technologies are advantageous owing to their low cost and efficiency, high throughput, excellent reproducibility, and ability to create complex geometries. The two major areas in which 3D printed in vitro tissue models have been applied are cancer research and drug screening systems. Table 47.1 summarizes bioprinting applications for in vitro biological systems.

Tumor Models

3D bioprinting of cells as tumor models are helpful for studying the interaction of immune and tumor cells and for screening new treatments [62]. Xu and Celli et al. introduced a 3D in vitro cancer model using human fibroblasts and ovarian cancer cells on a Matrigel matrix; they showed precise and reproducible control over the cell density and spacing compared with manual ejection by micropipettes [63]. Snyder et al. introduced a similar model in 2011 with human hepatic carcinoma cells and mammary epithelial cells. Cells and the microfluidic device were printed to test the radiation shielding of the prodrug amifostine. Radioprotective benefits for the liver were seen in their in vitro model [64]. Huang et al. examined tumor cell migration in a honeycomb structure with different channel widths (25, 45, and 120 μm) to mirror those of natural blood vessels [65]. HeLa and 10T1/2 cells were seeded within the device and were evaluated in the different channel sizes. HeLa cancer cells showed less morphological changes between channel sizes than did 10T1/2 cells and migrated at higher rates as the channel size decreased. Also looking at bioprinted HeLa cells, Zhao et al. examined the cell response after extrusion in a gelatin−alginate−fibrinogen hydrogel compared with a 2D culture model. Cells in the 3D model showed higher proliferation, matrix metalloproteinase expression, and chemoresistance [28]. Hribar et al. used 3D projection printing to create concave PEG structures that formed and maintained breast cancer spheroids for long-term culture [66]. The breast cancer spheroids exhibited necrotic, hypoxic cores, which are key components of the tumor in vivo microenvironment.

TABLE 47.1 Applications of Bioprinting In Vitro Biological Systems

Tissue/Organ	Bioprinting Method	Cell Type	Encapsulation Material	Testing	Outcomes	References
Tumor/cancer	Pneumatic cell droplet patterning	Fibroblasts and ovarian cancer cells	Matrigel	Reproducibility and precision of cell density and spacing	Bioprinting methods showed improved performance compared with micropipette ejection	[63]
Tumor/cancer	Temperature controlled, pneumatic extrusion	Hepatic carcinoma and mammary epithelial cells	Matrigel	Radiation shielding capabilities of the prodrug amifostine	Amifostine provided radioprotection to cells, with greatest benefit seen in dual-cell model	[64]
Tumor/cancer	Projection stereolithography (UV exposure)	HeLa and 10T1/2	Poly(ethylene glycol) diacrylate	The effect of channel width on tumor cell and 10 T1/2 migration and morphology	Tumor cells showed increased migration speed and less change in morphology with smaller channel sizes compared with 10 T1/2	[65]
Tumor/cancer	Extrusion	HeLa	Gelatin−alginate−fibrinogen	Viability, proliferation, MMP expression, and chemoresistance versus 2D culture	3D cell culture increases cancer cell proliferation, MMP expression, and resistance to chemotherapy	[28]
Tumor/cancer	Continuous 3D projection (with nonlinear exposure)	Breast cancer cells	Poly(ethylene glycol)	Long-term culture and validation of breast cancer spheroids	Spheroids showed hypoxic cores and signs of necrosis, key features of tumor environment	[66]
Liver	Extrusion	Hepatocytes	Alginate	Validation of liver cell activity and metabolic performance	Cells were viable, proliferative, synthesized urea, and metabolized 7-ethoxy-4-trifluoromethyl coumarin to 7-hydroxy-4-trifluoromethyl coumarin	[67,68]
Bacterial Infection	Inkjet	*Escherichia coli*	Alginate	Treatment of *E. coli* with several common antibiotics	Similar results to current low-throughput, less reproducible, and more expensive methodologies	[69]
Brain	Extrusion and subsequent dissolution of sacrificial resin	Mouse brain endothelial cells	Type 1 collagen microchannels	Model validation with transendothelial permeability measurements and hyperosmotic mannitol disruption test	Permeability decreased over 3 weeks of culture and then recovered over 4 days after hyperosmotic mannitol disruption	[70]
Lung	Extrusion	Endothelial and epithelial cells	Matrigel	Cell viability, distribution, morphology, and permeability compared with manually placed cells	Printed constructs resulted in more homogeneous cell distributions, proper cell morphologies, lower permeability, and similar viability	[71]

3D, three-dimensional; *MMP,* matrix metalloproteinase.

Drug Screening Systems

The number of in vitro drug screening systems has increased immensely in both quality and quantity, ranging from hepatic cells suspended in a microfluidic device to integrated, multiple-tissue, body-on-a-chip systems. Chang et al. used direct cell writing bioprinting to create a 3D microorgan housed using soft lithographic micropatterning [67]. Alginate-encapsulated hepatocytes printed in the microfluidic device were viable and proliferated and were capable of synthesizing urea. This work was furthered by infusing a hepatocyte containing a microfluidic device with 7-ethoxy-4-trifluoromethyl coumarin, which was metabolized into 7-hydroxy-4-trifluoromethyl coumarin, mimicking the in vivo behavior of the liver [68]. Rodriguez-Devora et al. developed an inexpensive drug-screening platform via inkjet bioprinting deposition. *Escherichia coli* were printed in an alginate solution with different antibiotic droplets patterned on the cells, resulting in similar bacteria inhibition compared with the current screening process [69]. An in vitro model of the blood–brain barrier was developed by Kim et al. Mouse brain ECs were cultured within an array of type I collagen microchannels fabricated using microneedles on a 3D printed frame [70]. The model was validated by measuring transendothelial permeability and a disruption experiment by hyperosmotic mannitol. In addition to the blood–brain barrier, an air–blood barrier was developed to model the lung. Horváth et al. developed a model by bioprinting epithelial cells and ECs separated by a basal membrane layer [71]. The bioprinted model was more reproducible and had thinner cell layers than that which could be manufactured using traditional manual methods.

In addition to these already successful in vitro models, several areas for future work stand out in this new field. Work is in progress to incorporate an array of tissue types into the same drug screening platform. Bioprinted in vitro models are also in good position to evaluate gene therapy techniques; however, standardized model systems and industry standards are needed to facilitate comparison across studies. The use of these in vitro models show promise in increasing our understanding of biology, disease progression, organ cross-talk, and many other areas as the field progresses.

Tissue Engineering Applications

Bioprinting has been used in the laboratory to fabricate constructs targeting nearly every tissue types in the body. Although clinical implantation is still rare in this relatively new technology, there have been many successes in vitro and in vivo. Highly detailed, anatomically correct, and patient-specific tissue constructs have been fabricated for a number of tissues and organs. A wide range of cells has been shown to maintain viability, gene expression, and functional capabilities after the printing process. Various stem cells have demonstrated the ability to preserve their differentiation potential and also have been directed by various cues applied during the printing process [72,73]. This section will highlight a select few of the many tissue-specific regenerative medicine applications that have been studied with bioprinting technologies.

Bone

Bone regeneration is a natural target application for bioprinting given the importance of anatomical structure to its in vivo function. Conventional 3D printing technologies are in use clinically as patient-specific metal implants [74]. Bioprinting offers a unique and promising alternative to bone grafting because of the wide variety in anatomic location, defect size, and patient-specific morphology for bone pathologies [75,76]. The advantage of bioprinting is especially apparent for bone defects, which also feature a significant cosmetic function such as in craniofacial reconstruction [77]. Fedorovich et al. printed a mixture of Matrigel and alginate hydrogels with endothelial progenitor cells and mesenchymal stem cells (MSCs) [78]. The constructs were implanted subcutaneously in immunodeficient mice. The researchers were able to demonstrate that incorporating biphasic calcium phosphate microparticles caused the MSCs to differentiate into an osteogenic lineage and caused bone formation within 6 weeks after implantation. Phillippi et al. used inkjet bioprinting to pattern bone morphogenetic protein 2 on primary muscle-derived stem cells on fibrin-coated coverslips [79]. The stem cells differentiated into an osteogenic lineage even under myogenic differentiation media conditions. Keriquel et al. demonstrated in situ bioprinting by delivering nanoscale hydroxyapatite and osteoblasts into mouse calvarial defects, with positive outcomes [80]. Using CT scanning, Yao et al. were able to print anatomically accurate PCL–hydroxyapatite mandible scaffolds that supported physiological loads [81]. Wang et al. examined the degradation profile of printed poly(propylene fumarate) scaffolds as it pertained to pore size, porosity, and mechanical properties [82]. They also developed a novel test for cytotoxicity of the degradation products and determined the scaffolds to be suitable for bone tissue engineering applications. To incorporate biological

materials with bioprinting technology, Pati et al. cultured MSCs onto a printed PCL−PLGA−β-tricalcium phosphate (TCP) scaffold [83]. The cells deposited ECM during a brief culture period, after which the scaffold was decellularized. In vivo, the ECM-enriched scaffolds induced greater bone formation than did unadorned scaffolds of the same composition.

Many limitations exist, including for large-sized defects and in high−load bearing applications. Lack of perfusion and neovascularization prevent large defects from being treated with bioprinting strategies; further research is needed in this area [84]. In addition, the discovery of new bioprinting-compatible materials and unique structural designs could increase maximum load-bearing applications for these constructs. More work is also needed to match the degradation profiles of scaffolding materials closely with those of the bone remodeling rate. New bone formation is obstructed if the scaffolding material degrades too slowly, but the defect site is left without a load-bearing material if degradation occurs too quickly, damaging nearby tissue. Altogether, bone tissue is one of the more promising target tissue applications for bioprinting owing to its many advantages relative to other tissue engineering strategies and the natural ability of bone to remodel in vivo (Table 47.2).

Cartilage

Articular cartilage is imperative to reducing friction and absorbing compressive forces in load-bearing joints with little to no capacity for self-regeneration. Current cartilage tissue engineering strategies are insufficient for reproducing tissue that is equivalent to healthy cartilage [85]. However, there has been greater interest in the zonal differences found in cartilage matrix and cellular composition [86]. Bioprinting presents an appealing tool for constructing stratified scaffolds, especially in patient-specific size and shape of individual lesions [87]. Gruene et al. used laser-induced forward transfer to generate MCS grafts, showing good cell viability, density, and functionality [88]. MSCs were able to differentiate into osteoblasts and chondrocytes and the graft maintained good structural integrity in vitro. Cui et al. loaded chondrocytes into poly(ethylene glycol) dimethacrylate hydrogel and inkjet bioprinted them into an osteochondral plug [89]. The implant had mechanical and biochemical properties similar to native cartilage, and Safranin-O staining revealed good integration with surrounding cartilage tissue. The same group also used their experimental setup to investigate the effects of fibroblast growth factor 2 and transforming growth factor β1 on cartilage generation. Samples were cultured up to 4 weeks, and the highest GAG content was found for samples containing both growth factors, suggesting a synergistic effect between increased cell proliferation and increased chondrogenic phenotype expression [90]. To address some of the limitations of bioprinting, Xu et al. alternated between inkjet-bioprinted layers of rabbit elastic chondrocytes suspended in a fibrin-collagen hydrogel and electrospun PCL [22]. The construct formed cartilage-like tissues in vitro and in vivo demonstrated by type II collagen and GAG deposition. In addition, the scaffolds with electrospun layers showed improved mechanical properties compared with those that were only bioprinted.

In particular, engineering the external ear has been a notably successful area of bioprinting cartilage tissue. The ear is almost completely avascular and aneural, it has a complex geometry, and it serves a largely aesthetic function, which places a greater emphasis on individualizing each prosthetic to the specific patient. Mannoor et al. developed a bionic ear that can translate sound waves into an electrical output [91]. The scaffold was extrusion bioprinted with sodium alginate, silver nanoparticles, and chondrocytes in an ear-shaped geometry around the conductive, sound-translating coil. Lee et al. extrusion printed PCL with PEG as a supporting sacrificial layer [92]. Chondrocytes and adipocytes were differentiated from adipose-derived stromal cells, encapsulated in alginate hydrogel, and dispensed into their respective regions. After 7 days in in vitro culture, immunostaining analysis confirmed chondrogenesis and adipogenesis. We also applied the extrusion-based bioprinting to fabricate a complex shape by making human-sized ear cartilage tissue construct (Fig. 47.5A) [47]. After implantation, the printed ear shape was well-maintained, with cartilage tissue formation upon gross examination. Histological analyses showed the tissue formation of cartilage tissue as confirmed by GAG and collagen type II staining. Quantitatively, GAG content increased over time, reaching 20% of that of native ear GAG content at 2 months after implantation.

At this stage, the next challenge for bioprinting as a means for cartilage regeneration is to conduct translational studies. Few in vivo studies have been conducted. The long-term stability of bioprinted cartilage constructs has yet to be demonstrated and no studies have compared these strategies with practices used clinically. However, research in cartilage bioprinting is growing exponentially and exhibits many promising results for the future.

TABLE 47.2 Three-Dimensional Bioprinting Technologies for Tissue Regeneration Applications

Tissue/Organ	Testing Model	Printing Method	Cell Type	Bioink	Outcomes	References
Bone	In vitro viability and differentiation studies	Extrusion	Endothelial progenitor and multipotent stromal cells	Matrigel and alginate hydrogels	Viability and differentiation capability were unaffected by printing process, and the two distinct cell populations were maintained within a single scaffold	[78]
	In vitro differentiation studies	Inkjet	Primary muscle-derived stem cells	Fibrin	Incorporation of bone morphogenetic protein-2 caused spatially controlled osteogenic lineage differentiation even in myogenic media conditions	[79]
	In situ bioprinting	Laser	Osteoblasts	Glycerol and n-Ha slurry	Successful in situ bioprinting into mouse calvarial defects with minimal side effects	[80]
	In vitro	Extrusion	–	Polycaprolactone-hydroxyapatite	Reconstructed from CT scans, anatomically accurate and supportive of physiological loads	[81]
	In vitro degradation, mechanical, and cytotoxicity	Laser	Fibroblasts	Poly(propylene fumarate)	Scaffolds maintained their mechanical stability, and degradation products did not induce significant cell death	[82]
	In vitro and in vivo bone formation	Extrusion	Mesenchymal stromal cells	PCL/PLGA/B-tricalcium phosphate	Scaffolds which were decellularized after brief culture period induced greater bone formation in vivo	[83]
Cardiac Muscle	In vitro	Extrusion	Spheroids of HUVECs and cardiac cells	Type 1 collagen	Viable cells, fusion and beating at 70 h with early signs of vascularization	[98]
	In vitro	Inkjet	Cardiomyocytes	Alginate	Viability in thickness as high as 1 cm and contraction was observed at macroscopic and microscopic levels	[99]
	In vivo cardiac infarct patch	Laser	HUVECs and human MSCs	Polyester urethane urea	Increased function and vessel formation compared with cell-only treatment 8 weeks postinfarction	[100]
	In vitro	Extrusion	Cardiac-derived cardiomyocyte progenitor cells	Alginate	Cells demonstrated viability, phenotypic cardiac expression, and ability to migrate from hydrogel	[101]
Cardiac valve	In vitro	Extrusion	Porcine aortic valve interstitial cells	PEGDA and alginate	Anatomical accuracy was confirmed; a range in mechanical properties was obtainable by varying the concentrations of the hydrogels	[6,103,104]
Cartilage	In vitro	Laser	MSCs	None	Good structural integrity and osteoblast and chondrogenic differentiation	[88]
	In vitro	Inkjet	Articular chondrocytes	PEGDMA	Similar mechanical and biochemical properties of native cartilage and good integration with surrounding tissue; fibroblast growth factor 2 and transforming growth factor β-1 synergistically improved GAG deposition	[89,90]

Continued

TABLE 47.2 Three-Dimensional Bioprinting Technologies for Tissue Regeneration Applications—cont'd

Tissue/Organ	Testing Model	Printing Method	Cell Type	Bioink	Outcomes	References
	In vitro and in vivo	Inkjet	Rabbit elastic chondrocytes	Fibrin–collagen	Combined PCL electrospinning and bioprinting technique facilitated type 2 collagen and GAG deposition with improved mechanical properties	[22]
	In vitro	Extrusion	Chondrocytes	Alginate	Biomimetic ear could translate sound waves into an electrical signal and coexist with viable chondrocytes	[91]
	In vitro	Extrusion	Chondrocytes and adipocytes	PCL and PEG (sacrificial) and alginate (encapsulation)	Chondrogenesis and adipogenesis confirmed by immunostaining	[92]
Skin	In situ bioprinting	Extrusion	Amniotic fluid derived stem cells	Fibrin–collagen	Amniotic fluid–derived stem cells outperformed both MSCs and acellular graft	[105]
	In vitro and in vivo	Laser	Fibroblasts and keratinocytes	Collagen	Early indicators of stratum corneum formation and blood vessels after 11 days	[106,107]
	In vivo	Inkjet	Fibroblasts, keratinocytes, and microvascular endothelial cells	Collagen	10% improvement of wound contraction compared to allogeneic skin substitute	[108]
Bone–cartilage	In vitro and in vivo	Extrusion	MSCs and chondrocytes	Alginate	Distinct tissue regions were found after 21 days in culture and 6 weeks after subcutaneous implantation	[113]
	In vitro	Extrusion	Osteoblasts and chondrocytes	Type 1 collagen and hyaluronic acid	Cells showed better proliferation, migration, and function on hydrogels made from their native ECM and performed well in 14-day coculture	[37]
Muscle–tendon	In vitro	Extrusion	Myoblasts and 3T3 fibroblasts	PCL & Polyurethane	Cells were viable after a week in culture and scaffolds showed an appropriate trend in mechanical properties	[96]
Pancreas	In vivo	Extrusion	INS1E β/islets	Alginate and gelatin	Scaffolds were formed and embedded while maintaining cell viability and morphology	[123]
Adipose	In vitro	Laser	Adipose-derived stem cells	Alginate	Cells maintained viability, differentiation potential, and adipogenic gene expression after 10 days	[121]
Neural	In vivo	Extrusion	Bone marrow MSCs and Schwann cells	–	Grafts underperformed autograft controls, but provide a proof-of-concept for future work	[114]
	In vitro	Inkjet	Retinal ganglion cells and glia	–	Good cell viability and growth properties of cells was found after printing	[115]
	In vitro and in vivo	Laser	Neuronal, Schwann, and dorsal root ganglion cells	PEG	After 3 weeks, the nerve guide supported reinnervation across a 3-mm gap equal to that of an autograft	[116]

CT, computed tomography; *FGF-2*, fibroblast growth factor-2; *GAG*, glycosaminoglycan; *HUVECs*, human umbilical vein endothelial cells; *MSCs*, mesenchymal stromal cells; *n-Ha*, nano-hydroxyapatite; *PCL*, polycaprolactone; *PEG*, poly(ethylene glycol); *PEGDA*, poly(ethylene glycol) diacrylate; *PEGDMA*, poly(ethylene glycol) dimethacrylate; *PLGA*, poly(lactide-co-glycolide).

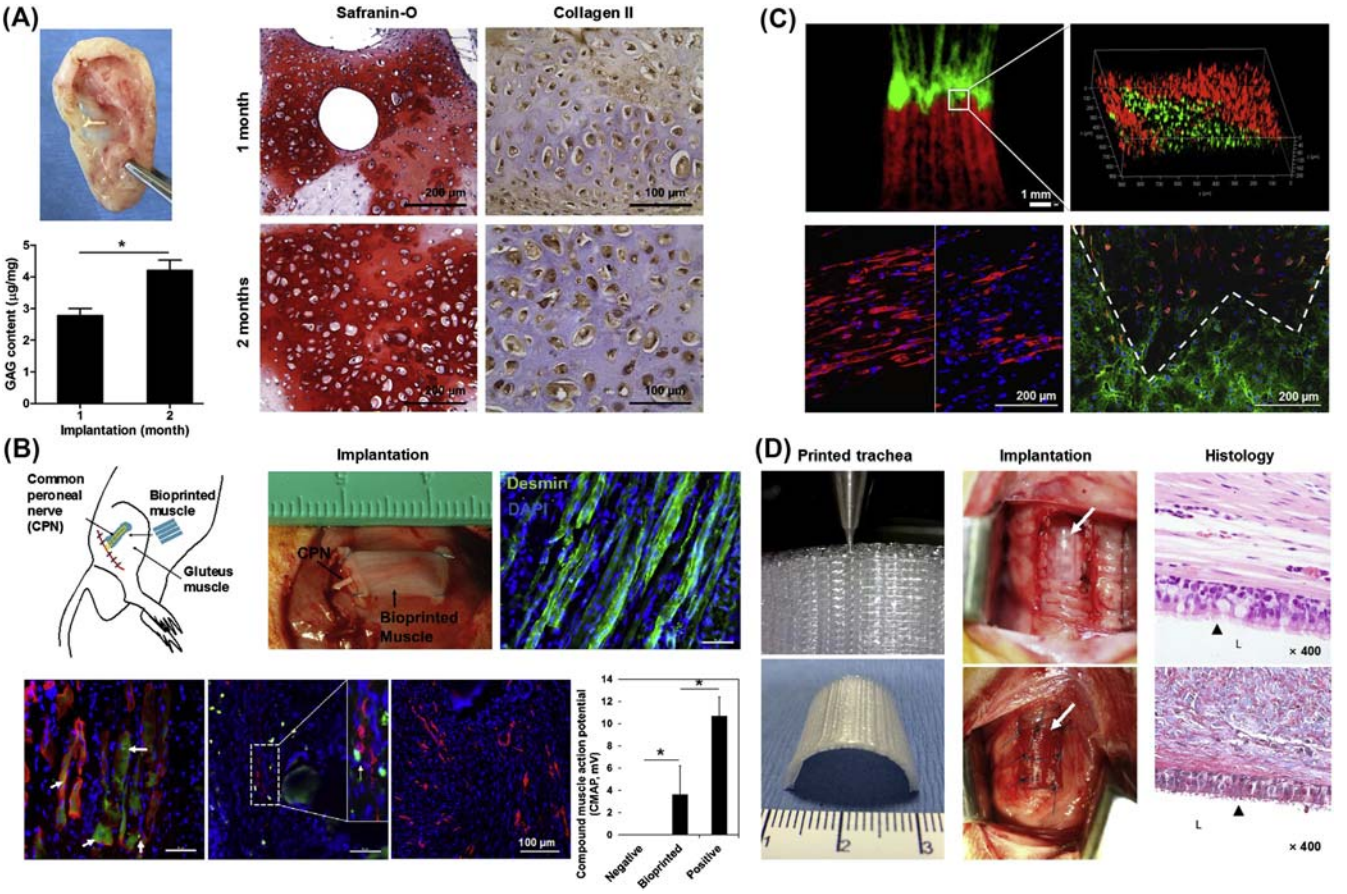

FIGURE 47.5 (A) Bioprinted ear construct: The shape was well-maintained, with substantial cartilage formation upon gross examination. Histological and immunohistochemical analyses showed the typical cartilage tissue formation [47]. (B) Bioprinted organized muscle construct: The retrieved muscle constructs showed well-organized muscle fiber structures, the presence of acetylcholine receptor clusters on the muscle fibers (myosin heavy chain (MHC+) and α-bungarotoxine (α-BTX+)), as well as nerve (neurofilament) contacts with α-BTX+ structures within the implants at 2 weeks after implantation [47]. (C) Bioprinted muscle–tendon unit (MTU): fluorescently labeled dual-cell printed MTU constructs (*green*: DiO-labeled C2C12 cells; *red*: DiI-labeled National Institutes of Health/3T3 cells; *yellow*: interface region between green and red fluorescence) [96]. (D) Bioprinted tracheal construct: Ingrowth of ciliary respiratory epithelium from the normal region was observed in the lumen of the bioprinted construct. Typical morphologies of respiratory mucosa and pseudocolumnar ciliary epithelium with goblet cells were well-developed at 8 weeks after implantation [120]. *DAPI*, 4,6-diamidino-2-phenylindole; *L*, lumen.

Skeletal Muscle and Tendon

The organized ultrastructure of skeletal muscle is required for muscle contraction and force generation [93]. Because 3D bioprinting mechanism enables control of the spatial organization of cell-laden bioinks, we were able to fabricate highly oriented, muscle-like bundles to engineer skeletal muscle construct (Fig. 47.5B) [47]. The printed, aligned cellular construct began stretching along the longitudinal axis at 3 days in culture, and the constructs underwent compaction from polymeric pillars, keeping the fibers taut during differentiation. The aligned muscle fiber-like structures were observed at 7 days in differentiation medium condition. This bioprinted skeletal muscle construct maintained the tissue organization, followed by tissue maturation and host nerve integration in rats. The results demonstrate that the 3D bioprinting is capable of producing promising the structural and functional characteristics of skeletal muscle constructs in vitro and in vivo.

Tendon has a hierarchical architecture, and tenocytes are aligned along with a dense collagen fibrous structure [94]. To mimic these structural characteristics of the tendon, an electrohydrodynamic jetting printing was introduced to generate a tubular-shape, multilayered tendon construct with highly porous, oriented microscale PCL fibers [95]. The cultured human tenocytes on the bioprinted structure showed a highly cellular orientation, metabolism, and type I collagen expression. 3D bioprinting technologies are particularly useful for composite tissue constructs such as muscle–tendon. We used our integrated tissue and organ printing system to print four different components to fabricate a single integrated muscle–tendon unit (MTU) construct (Fig. 47.5C)

[96]. The printed MTU construct was composed of mechanically heterogeneous polymeric materials that were elastic (polyurethane [PU]) on the muscle side and relatively stiff (PCL) on the tendon side, in addition to having a tissue-specific distribution of cells with C2C12 myoblasts on the muscle side and National Institutes of Health/3T3 fibroblasts on the tendon side. Results showed that cells were printed with high cell viability and cellular orientation as well as increased musculotendinous junctional gene expression. It was demonstrated that 3D bioprinting technology enabled a 3D heterogeneous tissue construction with region-specific biological and biomechanical characteristics.

Cardiac Tissue and Heart Valves

The heart is a complex organ in both shape and tissue organization, both of which are difficult to replicate by other fabrication methods. The ability to control the distribution of different cell types and growth factors spatially make bioprinting an attractive option for cardiac engineering, although only proof-of-concept successes have been accomplished thus far [97]. Jakob et al. were able to use extrusion-based bioprinting to pattern spheroids of HUVECs and cardiac cells on collagen [98]. The cells proved viable after the process; they fused at 70 h into a beating tissue and showed early signs of forming vascularization. Using inkjet bioprinting, Xu et al. printed a half heart shape (with two connected ventricles) with cardiomyocytes encapsulated in an alginate hydrogel [99]. Cell viability was preserved in constructs as thick as 1 cm owing to the designed porosity within the structure, and contraction was observed in vitro at both the microscopic and macroscopic levels. Instead of targeting whole-heart reconstruction, Gaebel et al. developed a cardiac patch for regeneration after cardiac infarction [100]. Using laser-induced forward transfer, HUVECs and human MSCs were patterned on polyester urethane urea and transplanted into the infarct zone. After 8 weeks, increased vessel formation and function were found, compared with a control treatment of bioprinted cells alone. A patch by Gaetani et al. used human cardiac-derived cardiomyocyte progenitor cells; they were extrusion-bioprinted in a sodium alginate mesh pattern [101]. They demonstrated cell viability, phenotypic expression of cardiac lineage, and the ability to migrate from the alginate, which suggested that bioprinting can be used to define cardiac cell delivery.

Patients with heart valve failure must receive a replacement valve that can be mechanical requiring a lifetime of anticoagulant treatment, or biological, which typically fails within 10–20 years [102]. Bioprinting has gained momentum as a potential heart valve fabrication strategy to mimic the complex geometry and nonhomogeneous material makeup, mechanical properties, and cell distributions that naturally occur in heart valves [102]. Hockaday et al. used a dual ionic and physical cross-linking hydrogel strategy by using a PEGDA sodium alginate composite [6]. The printing accuracy of the aortic valve root wall and trileaflets was confirmed via microCT scanning. By varying PEGDA and alginate concentrations, elastic moduli were found to range from 1.5 to 5.3 kPa. Porcine aortic valve interstitial cells were seeded and cultured on the scaffold for 21 days with nearly 100% viability. Later studies by the same group printed the cells directly within the hydrogel, as opposed to seeding the scaffolds afterward, also with good geometric accuracy, cell viability, and mechanical properties [103,104]. Although these studies are far removed from use in the clinic, they demonstrate that bioprinting technology is amenable to cardiac tissue regeneration and open the door for many future studies focused on improving the current methodology and outcomes.

Skin

Bioprinting is an excellent technology for depositing distinct layers. It has been used in an attempt to mirror the layers of native skin, and research in this area has increased significantly. Skardal et al. performed direct in situ printing of amniotic fluid–derived stem cells suspended in the fibrin–collagen hydrogel [105]. Compared with an acellular graft and an MSC graft, amniotic fluid–derived stem cells showed increased microvessel density and capillary diameter. Laser-assisted bioprinting has been used to embed fibroblasts and keratinocytes in collagen [106]. Histology revealed a high density of both cell types and the expression of laminin protein. The same group grafted their construct onto mice and reported early indicators of stratum corneum formation and blood vessels after 11 days [107]. Yanez et al. printed keratinocytes and fibroblasts in collagen as well, but they included human microvascular ECs [108]. When implanted onto the backs of mice and compared with allogeneic skin substitute as a control, wound contraction improved by 10% and histological results appeared similar to those of normal skin. Sweat glands and hair follicles remain elusive, as does commercial and regulatory viability [109]. Nonetheless, skin bioprinting has shown many encouraging successes, and the clinical bioprinting of skin appears to be an impending reality [20].

Other Tissue Types

Many other tissue types have been targeted with bioprinting technology, albeit to a lesser extent than those discussed thus far. This could be caused by a lesser clinical need, a higher difficulty of tissue engineering in general, or a poor match between the advantages of bioprinting and the necessary components for regenerating that tissue. Composite tissues are a major challenge facing regenerative medicine. No organ in the body is completely isolated, and many tissues such as tendons have specific and functional interfaces with other tissue types. Bioprinting is uniquely positioned to address this problem by spatially directing the placement of different cell types, growth factors, and biomaterials [110–112]. Fedorovich et al. extrusion bioprinted MSCs with hydroxyapatite, β-TCP, and biphasic calcium phosphate particles in alginate for one section of the scaffold and chondrocytes in alginate for the other [113]. Distinct tissue formation was found after 21 days in a mixture of osteogenic and chondrogenic media culture as well as after 6 weeks of subcutaneous incubation in vivo. Park et al. bioprinted osteoblasts in collagen I hydrogel and chondrocytes in HA hydrogel with good results after 14 days in vitro, in the process showing that the cells performed better on hydrogels made from their native ECM [37]. Finally, Merceron et al. targeted the MTU using PCL and 3T3 fibroblasts for the tendon zone and PU and myoblasts for the muscle zone [96]. Cells were viable after 7 days, and the scaffold showed appropriate trends in mechanical properties.

Neural tissue has also been addressed by bioprinting. Owens et al. extrusion bioprinted a nerve graft containing bone marrow MSCs and Schwann cells [114]. The grafts were implanted for 10 months in a rat sciatic nerve injury model with autograft controls. The researchers concluded that that bioprinting was a promising approach to nerve grafting. Retinal ganglion cells and glia were piezoelectric inkjet bioprinted by Lorber et al., and the results showed good cell viability and growth-promoting properties in vitro [115]. Pateman et al. used a microsterolithographic technique to print PEG-based nerve guides for nerve repair [116]. In a 3-week, common fibular nerve injury mouse model, the nerve guide was capable of supporting reinnervation across a 3-mm injury with results similar to that of an autograft.

Trachea is mainly composed of tightly stacked cartilage rings and respiratory mucosa in the luminal surface. Several synthetic implants have been used to reconstruct tissue defects [117–119]; however, these implants have been limited in their ability to mimic the tracheal functions biologically and biomechanically. We developed a biomimetic tracheal construct using a 3D bioprinting approach that could reconstruct a partial tracheal defect in a rabbit model (Fig. 47.5D) [120]. The printed tracheal PU constructs provided excellent structural characteristics. In the rabbit tracheal defect model, the printed PU constructs maintained the biomechanical function of the trachea, whereas the microscale porous architecture in the construct allowed cellular infiltration for the biological integration with host tracheal tissue. Moreover, the printed PU scaffold provided a proper microenvironment to facilitate the resurfacing of the ciliated respiratory epithelium and the ingrowth of connective tissue with microvasculature.

Adipose tissue is not often targeted by regenerative medicine strategies, but Gruene et al. [121] laser bioprinted adipose-derived stem cells encapsulated in alginate. They proved that the cells maintained their viability, differentiation ability, and adipogenic gene expression after 10 days in vitro culture. Preliminary progress has also been made in several more complicated organs such as the intestine [122] and pancreas [123]. This section briefly examined the application of bioprinting to specific tissue types. Many studies were excluded because of space constraints; several tissue types that have been explored via bioprinting have not been covered here.

CONCLUSIONS AND FUTURE PERSPECTIVES

The ultimate goal of tissue engineering and regenerative medicine is to reduce patient morbidity and mortality while improving quality of life by producing patient-tailored tissue constructs that induce tissue regeneration. 3D bioprinting technologies hold great promise for achieving this goal. The focus on replicating complex and heterogeneous tissue constructs continues to increase as 3D bioprinting technologies progress. Progression from single, simple tissues such as skin, bone, and cartilage, to organized contractile tissues such as skeletal muscle and cardiac tissue, to composite tissues such as osteochondral tissue and MTUs, and finally to robust organs such as the kidney and heart are under way.

A novel bioink system needs to be developed to improve printability with high-resolution capability and structural maintenance. The availability is limited of biomaterials, including hydrogels and polymers, in 3D bioprinting that can serve as cell delivery bioinks and supporting structures, but which also provide biological properties and mechanical and structural support. Advances in biomaterials depending on 3D bioprinting mechanisms

are necessary for long-term success in tissue engineering applications. An approach that uses the decellularized ECM can provide a tissue-specific microenvironment to the cells. Decellularized ECM-based bioinks are still the closest biological microenvironment that mimics in vivo conditions; thus, tissue-specific ECM-based bioinks are capable of providing critical cues for targeted cell engraftment, survival, and tissue formation.

There must be an increase in knowledge about biological, anatomical, and physiological aspects of complex tissues and organs. In particular, the development of in vitro 3D tissue models to study tissue-specific functions in the body will require a better understanding of morphological, structural, and functional units in tissues or organs. Moreover, the well-known limitation to building a large-scale tissue construct is vascularization in the construct. 3D bioprinting may have a unique capability among the various tissue engineering technologies to overcome this limitation. A few groups have made progress toward printing vascular structures; however, integrating functional microvascular structures into tissue or organ-like constructs has not been accomplished. Approaches to using high porosity, angiogenic factors, and highly organized patterns of vascular cells may encourage vasculogenesis.

Bioprinting methods are able to construct 3D free-form shapes containing multiple cell types, biomaterials, and bioactive molecules, resulting in sophisticated tissue constructs that have the potential to replace damaged or diseased human tissues and organs. Although there is much work to be accomplished to advance these technologies toward successful clinical translation, our efforts will constantly contribute to producing clinically applicable tissue constructs until 3D bioprinting is able to improve the lives of patients.

List of Abbreviations

3D Three-dimensional
β-TCP β-Tricalcium phosphate
CAD Computer-aided design
CAM Computer-aided manufacturing
CT Computed tomography
DNA Deoxyribonucleic acid
ECM Extracellular matrix
GAG Glycosaminoglycan
GelMA Gelatin methacrylate
HA Hyaluronic acid
HUVECs Human umbilical vein endothelial cells
MSCs Mesenchymal stem cells
PCL Poly(ε-caprolactone)
PEG Poly(ethylene glycol)
PEGDA Poly(ethylene glycol) diacrylate
PLGA Poly(D,L-lactic-*co*-glycolic acid)
UV Ultraviolet light

Glossary

Bioprinting The incorporation of biological materials into additive manufacturing techniques, either by directly depositing cells layer by layer or indirectly by three-dimensional printing biologically active materials for later use in cellular applications.
Cross-linking A chemical bond between two polymer chains that changes the overall properties of the material.
Electrospinning A method for producing fibers that uses electrical forces to draw out nanoscale threads of melted polymer material.
Extracellular matrix The environment secreted by cells that biochemically and structurally supports a cellular network.
Extrusion bioprinting Direct contact bioprinting mechanism that relies on pressure or displacement to force material through the syringes.
Fused deposition modeling An additive manufacturing technology that extrudes heated material layer by layer to create three-dimensional structures.
High-throughput screening Drug delivery process in which a lot of drugs or chemicals can be tested at a rapid pace.
Hybrid bioprinting Using multiple bioprinting mechanisms in one system to overcome the limitations of each mechanism.
Hydrogel A polymeric gel material in which the main component is water.
In situ bioprinting Bioprinting directly in vivo such as onto a skin wound or into a bone defect, as opposed to bioprinting separately and then surgically placing a scaffold into a defect.
Jetting bioprinting Originating from inkjet printers, this noncontact bioprinting mechanism uses pressure pulses to apply bioink in predetermined locations.
Laser-assisted bioprinting Bioprinting mechanism that uses a focused laser to generate high-pressure bubbles that propel cell-containing material onto a substrate.
Microfluidic device A device that is able to manipulate and control the flow of fluids on a microliter to picoliter scale.
Scaffold The material which acts in place of the extracellular matrix , providing a physical, three-dimensional environment for cells to attach, migrate, and proliferate.

Micropatterning Precisely controlling the cellular microenvironment on a substrate for the purposes of studying cell behavior.
Perfusion The process of oxygen and other vital nutrients being delivered from the bloodstream to tissues and cells.
Piezoelectric A ceramic crystal that creates an electric charge in response to an applied mechanical stress.
Printability The ability and usefulness of a particular material to be applied as a bioink.
Printing resolution The smallest dimension that can be controlled by a particular bioprinting system.
Spheroid A three-dimensional conglomerate of cells, often organized into a spherical shape.
Stereolithography Three-dimensional printing process in which liquid photopolymer is exposed above a perforated platform and then cross-linked by a UV laser forming the first layer. The platform then lowers exposing a new surface of liquid which the UV laser cross-links to form layer two.
Structural stability The ability of a printed construct to maintain its shape.

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CHAPTER

48

Biomineralization and Bone Regeneration

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DEVELOPMENT AND FRACTURE OF BONE

Bone Development

Normal bone formation takes place by two different processes: endochondral ossification and intramembranous ossification. The endochondral ossification process features a sequential formation and degradation of cartilaginous tissue. By contrast, intramembranous ossification occurs by direct differentiation of precursor cells into mature osteoblasts [1].

Limbs of the body undergo endochondral ossification whereas the flat bones in the skull are formed by intramembranous ossification. Although these two processes are regulated by many of the same signaling molecules, the means by which cells differentiate and form bone are vastly different. In intramembranous ossification, precursor cranial neural crest cells differentiate into osteoblasts, which are bone-forming cells [2,3]. By contrast, mesenchymal stem cells (MSCs) establish a cartilage template, which is subsequently mineralized to form cortical bone during endochondral ossification [4]. During embryonic development, specific embryologic zones are defined for the formation of precisely defined structures of cartilage and bone. These early processes are regulated by several signals including soluble growth and differentiation factors, as well as cell−cell and cell−extracellular matrix (ECM) interactions. During an initial commitment phase, cells that will form bone are committed in a defined time and space. After initial commitment, cells are then differentiated into the terminal and mature cell phenotypes that are needed to construct bone.

Fracture Healing

In some ways, bone fracture healing is similar to the process of initial bone development, as described earlier [5]. It involves the establishment of an environment that drives the differentiation of precursor cells to repair and replenish the tissue at the site of the injury. Fracture healing has several distinct features. After a fracture occurs, there is an immediate inflammatory response that recruits activated macrophages and polymorphonuclear neutrophils to the site of injury. The macrophages release multiple factors that stimulate the formation of a hematoma. Granulation tissue fibroblasts then proliferate to form a blastema. Osteoprogenitor cells are then recruited from the periosteum, surrounding soft tissue, and bone marrow at the site of the fracture. These cells differentiate into chondrocytes and osteoblasts to repair the fracture. Several soluble growth and differentiation factors influence this process. Mitogenic growth factors stimulate proliferation of precursor cells. They include fibroblast growth factors, insulin-like growth factors, and platelet-derived growth factors. Differentiation factors such as bone morphogenetic proteins (BMPs) induce the differentiation of precursors into osteoblasts and chondrocytes. Typically, differentiation follows a proliferative phase, so BMPs are often found in higher concentrations later in the healing process.

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PRINCIPLES OF BONE TISSUE ENGINEERING

For fractures to be healed successfully, a sufficient number of precursor cells must be present at the site of injury and they must be directed toward osteogenic differentiation. Cells can be recruited in vivo or implanted directly. Expression of signaling factors can be upregulated or they, too, can be applied directly. The signals must be given in appropriate time course and quantity to stimulate cell growth and differentiation in a controlled manner.

Although fractures can heal naturally without intervention, larger defects require clinical interventions to repair or regenerate bone. These are referred to as critical-size defects. The reference standard treatment of these defects is autologous bone grafting, a process by which donor bone tissue removed from the patient's hip is shaped according to the site of injury and surgically implanted into the defect site. These grafts contain viable cells, signaling factors, and a matrix that can support healing. However, the success of these grafts is variable, the surgical intervention has associated risks, and donor site morbidity has been reported as a complication of this treatment.

In this chapter, we provide an updated overview of the state of bone tissue engineering as a means for treating bone defects and disease [6]. Bone tissue engineering provides an alternative method to regenerate bone while eliminating some limitations associated with grafts. In tissue engineering, a precisely engineered scaffold can be combined with an appropriate osteoprogenitor cell type and relevant growth and differentiation factors [7]. Damaged tissue sites that reach critical sizes can have limited self-healing potential in terms of the availability of precursor cells and concentration of growth and differentiation factors, as well as by scarring or inflammation. Thus, delivery of cells and growth factors can boost the efficacy of bioengineered scaffolds for bone regeneration in these cases. Nucleotide and gene delivery [8] and immune modulation [9] are also being pursued in bone and bone regeneration research.

STEM CELLS IN BONE TISSUE ENGINEERING

Stem cells are defined cell populations with the capacity for self-renewal and the potential for differentiation to multiple cell fates [10]. In bone tissue engineering, adult MSCs, which are often derived from bone marrow, are the most common cell type used to regenerate bone. Other cell types frequently used in the field include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adipose-derived stem cells (ADSCs).

Mesenchymal Stem Cells

MSCs are a common cell source for bone regeneration therapies because they are easily obtained and amplified. They are a multipotent cell type and have low immunogenicity. In vivo, MSCs develop into cells that make up bone, cartilage, and fat. MSCs have been isolated from fetal blood, liver, and umbilical cord, but they are most frequently derived from bone marrow. In culture, they exhibit fibroblastic morphology. They easily adhere to plastic dishes and flasks and can be expanded in culture for several passages without loss of phenotype.

Osteogenic differentiation of MSCs can be stimulated in vitro by an induction media supplemented with dexamethasone, ascorbic acid (vitamin C), and β-glycerophosphate. Exposure to osteogenic media causes upregulation of alkaline phosphatase (ALP) activity and expression of two transcription factors that direct osteogenic differentiation, runt-related transcription factor 2 (Runx2) and osterix in MSCs during early stages of differentiation. During later stages, expression of matrix proteins including osteocalcin, osteopontin, and bone sialoprotein can be observed. Deposition of calcium within the ECM is also observed as osteogenic differentiation progresses (Table 48.1).

Adipose-Derived Stem Cells

Like bone marrow, white adipose tissue is mesodermally derived and contains endothelial cells, smooth muscle cells, and MSCs. The MSCs can be isolated from adipose tissue and expanded in culture. These cells (ADSCs) behave similarly in culture to MSCs obtained from bone marrow. They are multipotent and highly proliferative and can be obtained in large numbers with minimal invasiveness. ADSCs can be directed toward osteogenic differentiation with the same induction media used for MSCs. Given their similarities to bone marrow–derived MSCs, ADSCs are a clinically relevant cell source with potential for use in bone tissue engineering. However, compared with MSCs from other sources, the efficiency of osteogenic differentiation of ADSCs is low in both in vitro and in vivo

TABLE 48.1 Markers of Osteogenic Differentiation

Marker of Osteogenic Differentiation	Highest Expression During Stages of Osteogenic Differentiation	Function
Alkaline phosphatase (ALP)	Early	The function of ALP is not well-described; however, it is thought to have a role in promoting calcification of tissue [133].
Runt-related transcription factor 2 (Runx2)	Early	Runx2 is a transcription factor that activates osteogenic genes and has a role in cell fate determination of mesenchymal stem cell to osteoprogenitor cells [134].
Osterix	Early	Osterix is a transcription factor that acts downstream of Runx2 to direct osteogenic differentiation [135]
Osteocalcin	Late	Osteocalcin is the most abundant noncollagenous protein in bone extracellular matrix. It serves as a metabolic regulator of bone [136].
Bone sialoprotein (BSP)	Late	Extracellular matrix protein of bone that regulates the microenvironment of osteoblasts, hypertrophic chondrocytes, and osteoclasts. Regulates bone formation, mineralization, and turnover [137].
Osteopontin	Late	Similar to BSP, osteopontin is an extracellular matrix protein that regulates mineralization and bone matrix quality [138].

studies [11–13]. Therefore, many researchers have designed studies that aim to increase osteogenic differentiation of ADSCs by treating cells with osteogenic growth factors or introducing osteoconductive scaffolds [13].

Embryonic Stem Cells

ESCs are derived from fertilized embryos, typically donated from embryos that are not used after in vitro fertilization. These cells are grown in a plastic dish on a layer of feeder cells that maintain ESCs in their undifferentiated, pluripotent state. To differentiate ESCs, the cells are allowed to clump, forming embryoid bodies that can then be subjected to distinct culture conditions and develop into all three primordial germ layers. Direct differentiation of ESCs or differentiation of ESCs to an intermediate MSC stage can be used in tissue engineering applications [14]. Culture of committed cells is conducted in monolayer and supplemented with lineage-specific media conditions. Osteogenic differentiation media used to differentiate ESCs are the same combination of exogenous factors used for MSC differentiation. Although ESCs have greater capacity for self-renewal and can result in more tissue types compared with MSCs, ethical issues and complex culture requirements complicate their clinical and research use.

Nonetheless, human ESCs (hESC) or hESC-derived mesenchymal progenitor cells have been seeded onto an osteoconductive nanofibrous scaffold and subsequently treated with osteogenic factors. These culture conditions guide the osteogenic differentiation of hESC-derived cells in three dimension (3D) and demonstrate the potential role of hESCs in bone tissue engineering [14,15].

Induced Pluripotent Stem Cells

As an alternative to the controversial use of ESCs, iPSCs were developed to have the same capacity for self-renewal and pluripotency as ESCs. These cells are adult cells that have been reprogrammed to behave like ESCs. When iPSCs were initially described in 2006, researchers exposed mouse embryonic or adult fibroblasts to four factors under culture conditions used to maintain ESCs. The resulting cells had the same morphology, growth properties, and gene expression as ESCs and formed tumors of all three germ layers [16]. These cells were referred to as iPSCs.

The innovation of iPSCs was revolutionary to the field of stem cell biology, because it opened new areas of research into cell fate determination. It also opened the door to the use of iPSCs in tissue engineering. Several studies combined iPSCs or iPSC-derived cells with tissue engineering constructs to regenerate bone. In tissue engineering, one of the most appealing characteristics of iPSCs is that they originate from a patient with a tissue defect. The ability

to isolate and proliferate a patient-specific cell source would alleviate the risks in tissue engineering of allogenic graft rejection or disease transmission.

Several methods and several adult cell types are being used to generate iPSCs. The most common of these methods is exposure of cells to exogenous factors, genetic modification by viruses, and other methods of genetic alteration [17,18]. Researchers are pursuing methods that increase the efficiency of reprogramming and reduce the risk for tumor formation [17,19–22]. As methods improve to reprogram adult somatic cells, so does the variety of cell types that can be reprogrammed to iPSCs. In humans, dermal fibroblasts are among the most common cells used for iPSC generation. Other cell types that have been reprogrammed include dental and oral cells, cord blood cells, and peripheral blood cells [23].

Human and murine iPSCs show promise for osteogenic differentiation [24,25] and in bone tissue engineering [26–31]. Like other cell types, human iPSCs and cells derived from iPSCs can be seeded onto scaffolds and differentiated bone-forming osteoblasts.

SCAFFOLDS FOR BONE TISSUE ENGINEERING

Scaffolding Design Criteria

Scaffolds engineered for bone regeneration are designed to support cell adhesion, migration, proliferation, and differentiation [7,32–34]. Key features of a supportive architecture for bone tissue engineering scaffold include a controlled porous microstructure, high interconnectivity between pores, mechanical stability, a controlled degradation rate, and osteoconductive interaction with cells. The porous microstructure facilitates the ingrowth of cells and enhances the regeneration of tissue. Interconnectivity of pores allows for uniform cell seeding and nutrient exchange. Scaffolds are designed to degrade at controlled rates to match the rate of formation of new tissue. Finally, the structure and microenvironment of scaffolds are conducive to differentiation of precursor cells into osteoblasts and to support functional activities of osteoblasts in bone regeneration. In addition, it was found that a fibrous nanostructure could enhance osteoconductive characteristics of scaffolds for bone tissue engineering [35]. Nanofibers mimic the native ECM of bone and nanofibrous scaffolds support enhanced osteogenic differentiation compared with non-nanofibrous scaffolds. Nanofibrous scaffolds were shown to enhance osteogenic differentiation in vitro and heal critical size calvarial defects better in a rat model compared with solid-walled scaffolds [36,37].

To fabricate porous 3D scaffolds and microspheres for bone regeneration, a variety of processing techniques have been developed. These include solvent casting/particulate leaching [38,39], gas foaming [40,41], emulsion freeze-drying [42], electrospinning [43,44], rapid prototyping [45,46], and thermally induced phase separation (TIPS) [47–49]. Each of these methods has advantages and disadvantages that are discussed at length in several review articles [7,35,50–52]. This chapter will not provide an exhaustive description of processing techniques. Instead, it will focus on how scaffolding design can be used to improve bone tissue engineering with respect to porosity, interconnectivity, mechanical strength, elastic modulus, morphology, and surface properties.

Porous and Highly Interconnected Scaffolds

The porosity and interconnectivity of pores in scaffolds are important for uniform distribution of cells when seeded on a scaffold. A classical method to obtain porous scaffolds is the solvent casting/salt leaching method [38]. The process involves casting a mixture of polymer solution and salt (NaCl) into a mold. Subsequent drying of the mixture and leaching of the salt with water result in a porous structure. The pore size and porosity of the scaffolds fabricated using this method can be controlled by the particle size of the added salt and the salt-to-polymer ratio. This method produces scaffolds with limited interpore connectivity, which limits its value in tissue engineering.

To create a scaffold with highly interconnected pores and a spherical pore shape, paraffin spheres and sugar spheres were used a porogen [53,54]. The interconnectivity of the scaffold was finely tuned by changing the heat treatment time to bond the paraffin spheres. Meanwhile, changing the concentration of polymer solution, size of paraffin spheres, and number of casting steps controlled the porosity and pore size. Together, this approach allowed for the creation of porous scaffolds with high interconnectivity, which is critical to the uniformity of cell seeding and tissue ingrowth on the scaffold.

Nanofibrous Scaffolds for Bone Tissue Engineering

It is well-established that the ECM has a critical role in regulating cell behavior [55]. As previously noted, one of the key design features in bone tissue engineering has been the development of scaffolds with fibrous nanostructures that mimic native ECM and support cellular attachment, differentiation, and proliferation. Therefore, scaffolds were designed with nanofibrous structures that resembled the collagen fibers present in native ECM [56]. Collagen nanofibers were shown to have an integral role in cell adhesion, proliferation, and differentiation as early as the 1980s. To create poly-L-lactic acid (PLLA) scaffolds with nanofibers that resembled collagen fibers, a liquid–liquid phase separation technique was developed by integrating sugar sphere template leaching with phase separation [57]. The resulting nanofibrous PLLA (NF-PLLA) matrix featured fibers with diameters ranging from 50 to 500 nm, or within the same range of collagen fibers [49,53,58,59]. Fig. 48.1.

The combination of techniques to make porous materials and techniques to make nanofibrous materials produced scaffolds with biomimetic nanostructures and porous microstructures, which incorporate the advantages of both synthetic materials and natural structures for bone tissue engineering.

Nanofibrous materials have been successfully fabricated using electrospinning for applications in bone tissue engineering. Electrospinning uses an electric field to overcome the surface tension of polymer solutions. This causes the polymer to be ejected out of a needle to a conductive collector, resulting in fibers on the nanoscale to submicroscale [35]. Early electrospun scaffolds for bone tissue engineering were made of poly-ε-caprolactone (PCL) as the biodegradable polymer of choice [60–62]. Today, electrospun scaffolds are made from a variety of synthetic and natural polymers and can also be combined into composite scaffolds, as described in the next section. Several different electrospun scaffolds for bone tissue engineering have been described, including one method that used PLLA nanofibrous scaffolds that were produced by both thermally induced phase separation and electrospinning, and enhanced by surface calcium phosphate deposition for bone tissue engineering [63,64].

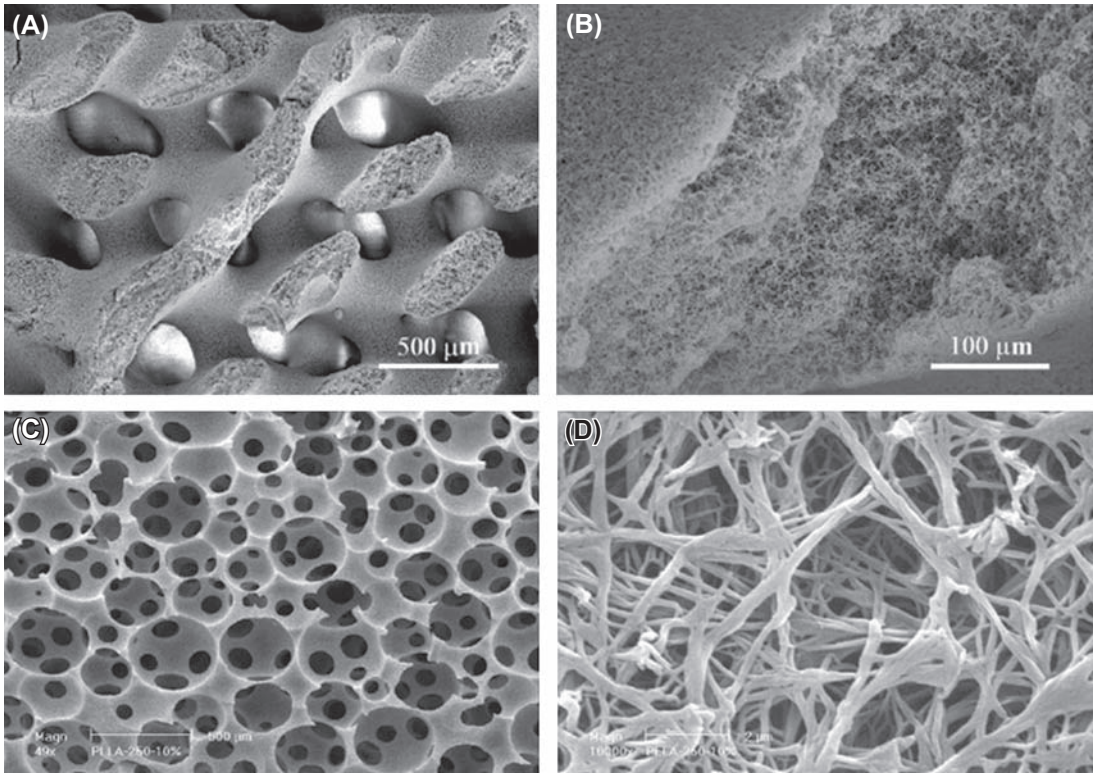

FIGURE 48.1 (A, B) Scanning electron micrographs of scaffolds fabricated using a spherical porogen (paraffin sphere) leaching technique: (A) poly-L-lactic acid (PLLA), (B) poly(lactic-*co*-glycolic acid) (85/15), and (C, D) PLLA scaffolds fabricated using a thermally induced solid - ± liquid phase separation technique in dioxane (C) or benzene (D). *(A, B) From Ma PX, Choi J-W. Biodegradable polymer scaffolds with well-defined interconnected spherical pore network. Tissue Eng 2001;7(1):23–33; (C, D) From Ma PX, Zhang R. Microtubular architecture of biodegradable polymer scaffolds. J Biomed Mater Res 2001;56(4):469–77; Ma PX, Zhang R, Xiao G, Franceschi R. Engineering new bone tissue in vitro on highly porous poly(alpha-hydroxyl acids)/hydroxyapatite composite scaffolds. J Biomed Mater Res (United States) February 2001;54(2):284–93.*

Composite Materials for Bone Tissue Engineering Scaffolds

The makeup of natural bone ECM is a combination of fibrous proteins, primarily collagen, upon which mineralized calcium is deposited [55]. In bone regeneration, researchers often attempt to replicate the natural ECM by synthesizing polymers from natural or synthetic building blocks. Because of intrinsic limitations of individual polymers, it is challenging to create a tissue engineering scaffold using a single material source that incorporates the fibrous microstructure of native ECM and supports sufficient mineralization. There are key limitations to each individual material, as no "perfect" single material exists for bone tissue engineering. Researchers therefore combine materials in various ways to form composite scaffolds that possess the advantageous qualities of each component material and better support the physical and biochemical requirements of bone regeneration. Integration of a mineral component into a fibrous scaffold can contribute to both the structural integrity and osteoconductive activity of the scaffold [35].

A variety of synthetic materials have been developed for bone tissue engineering, including PLLA, poly(lactic-*co*-glycolic acid) (PLGA), and poly(glycolic acid). Similarly, collagen, hydroxyapatite (HAP), decalcified bone matrix, chitosan, and other natural materials have been used in bone regeneration applications. The goal of composite scaffolds is to have a biocompatible, osteoconductive matrix with suitable mechanical properties to support bone regeneration.

One early composite matrix was made from combining synthetic polymer with ceramic through thermally induced phase separation. These scaffolds, made from highly porous poly(α-hydroxy acids)/HAP, had significantly improved physical properties (compressive modulus and yield strength) compared with pure polymer scaffolds. They also improved cellular ingrowth, increased osteoblast survival rate, and enhanced bone-specific gene expression [59,65,66]. Alternatively, coating a polymeric scaffold with apatite particles can produce a composite scaffold with the improved ability to deposit calcium in bone regeneration [66,67].

Other composites have been developed in an effort to combine the advantageous qualities of different synthetic and natural materials [68–70].

Injectable Scaffolds

Concurrent with the development of implantable 3D scaffolds for tissue engineering, researchers have sought to develop injectable scaffolds in the form of hydrogels, microspheres, or nanospheres.

Microspheres

An effort to fabricate microspheres with the same porosity and interconnectivity of 3D scaffolds yielded two new tissue engineering scaffolds. These particles retain the favorable characteristics of their implantable counterparts while having the potential for noninvasive treatment. The nanofibrous hollow microsphere (single pore) and the nanofibrous spongy microsphere (multiple pores) (NF-SMS) were created to mimic the physical characteristics of a 3D scaffold in an injectable format. Nanofibrous microspheres were made from linear PLLA create solid nanofibrous microspheres. By substituting linear PLLA with star-shaped PLLA in a surfactant-free emulsification technique, a hollow microsphere, with a single pore was developed [71]. By changing the number of arms on the star-shaped PLLA and altering its hydroxyl density, additional pores could be introduced, forming the NF-SMS [72]. These two novel microsphere scaffolds have shown promise for cartilage and dental tissue regeneration [71,73].

Other microsphere-based systems are used as cell carriers and/or drug carriers and are composed of synthetic, natural, or composite polymers [74–78]. These can be porous or nonporous and are used independently as tissue engineering platforms, or in combination with a 3D scaffold or hydrogel [79,80].

Hydrogels

Considerable research has been conducted into using hydrogels for bone regeneration. Hydrogels are polymer networks made of natural or synthetic components that are hydrophilic in nature and form viscous gels owing to 3D interactions between the polymer chains. These interactions can be either physical or chemical cross-linked between polymer chains. In bone tissue engineering, a number of hydrogels have served as a scaffold or used to deliver osteogenic drugs. An ideal hydrogel should be injectable so that it can be used to treat a bone defect noninvasively and fill abnormally shaped tissue defects. It should also be biocompatible and able to release osteogenic drugs over an extended time [81]. Controlled drug release remains a challenge for hydrogels because they are often prone to a burst release of drug within the first 24–48 h of treatment.

Hydrogels are often combined with other materials to form composite scaffolds for bone regeneration. PLLA/PCL nanoyarns were suspended in a collagen hydrogel to make a composite scaffold that showed benefits for in vitro differentiation of human MSCs [82]. In another study, 45S5 Bioglass was introduced into alginate, forming a composite hydrogel that demonstrated enhanced osteogenic potential with MC3T3-E1 osteoblast precursor cells [83]. A third study used a nanohydroxyapatite-reinforced chitosan hydrogel to stimulate osteogenic differentiation in vitro and bone regeneration in vivo [84]. These represent a small sampling of the many composite hydrogels being evaluated for bone tissue engineering.

Surface Modification and Functionalization of Scaffolds for Bone Regeneration

In an effort to increase the effectiveness of tissue engineering scaffolds, biologically active molecules can be attached to the surface or integrated within the scaffold. A specific molecule or peptide can be chosen to promote cell attachment and proliferation or direct cell differentiation [57,85,86]. For example, modification of poly(α-hydroxy acids) scaffold with gelatin enhanced osteoblast attachment, proliferation, and deposition of collagen [87]. Surface modification of a PLLA scaffold with calcium phosphate by electrodeposition enhanced osteogenic differentiation of MC3T3-E1 cells compared with cells attached to a control PLLA scaffold [64]. PEG hydrogels that are functionalized with integrin-specific peptides can enhance bone formation and vascularization when also treated with vascular endothelial growth factor [88]. Scaffolds can also be functionalized by the deposition of diamond nanoparticles. The addition of nanoparticles to a conventional scaffold can change the physical and chemical characteristics of the scaffold and enhance cell attachment and differentiation to support better bone formation [89]. Various additional methods and strategies for surface modification of scaffolds are being pursued [85,90].

Three-Dimensional Printed Scaffolds

3D printing technology is becoming increasingly capable of designing microenvironments and developing novel tissue engineering platforms. Many 3D printed scaffolds for bone regeneration have been developed that use many of the same natural, synthetic, and composite polymers discussed previously [91]. One approach to 3D printing scaffolds for bone tissue engineering is to print scaffolds that can be used to make vascularized bone grafts [92]. By varying the internal porosity of the scaffold or adding osteogenic and angiogenic factors such as zinc and silicon, researchers have attempted to increase the neovascularization of regenerated bone and simultaneously enhance the osteogenic differentiation of progenitor cells [93–95]. The successes and challenges of current strategies, descriptions of 3D printing methodologies, and clinical progress of 3D printing for bone tissue engineering are covered in depth in relevant reviews in the literature [96–98].

GROWTH AND DIFFERENTIATION FACTORS IN BONE TISSUE ENGINEERING

Bioactive molecules, hormones, and nucleic acids can be used to enhance the growth and differentiation of cells. The effective combination of these factors with tissue engineering constructs and drug delivery systems can improve the regeneration of bone both in vitro and in vivo.

Bone Morphogenetic Proteins

A group of proteins within the transforming growth factor-β superfamily, known as BMPs, have various roles in bone development, as well as the development of a multitude of tissues within the human body. Fifteen BMPs have been identified; of those, BMP-2 and BMP-7 show the most robust activity to induce bone formation. Both BMP-2 and BMP-7 have been used clinically in US Food and Drug Administration (FDA)-approved devices to treat a narrow range of severe bone defects.

BMPs act on bone-forming osteoblasts, preosteoblasts, and other precursor cells, including MSCs, ADSCs, iPSCs, and ESCs. Typically, BMPs form homodimers and bind with known BMP receptors to activate a cascade of signaling pathways that initiate differentiation and mineralization of cells and tissue. Some evidence of BMP-2/7 heterodimers having a stronger osteogenic effect than the respective homodimers has been published [99,100]. BMP-2 and BMP-7 act to stimulate osteogenic differentiation through intracellular phosphorylation of the smad

1/5/8 proteins. Upon phosphorylation, p-smad 1/5/8 proteins translocate to the nucleus and upregulates Runx2. BMPs can also activate other pathways in parallel, including Wnt signaling [101]. BMP-related signaling is generally divided into either smad-dependent or smad-independent, depending on which pathway is being described or studied.

The clinical use of BMP-2 began after its approval by the FDA in 2002 for the treatment of anterior lumbar interbody fusion within a threaded titanium tapered cage. The scope of its use expanded in 2004 to include tibial nonunions, and in 2007 for oral maxillofacial reconstruction. After the expanded use of BMP-2 for these clinical and other off-label applications, a number of serious side effects were observed and reported [102]. Inflammatory complications, including swelling of the cervical spine [103], radiculopathy [104], ectopic bone formation [105,106], osteoclast activation [107,108], urogenital events [109], and wound complications were reported [102].

For in vitro bone regeneration, BMP-2 and BMP-7 are applied in concentrations ranging from 50 to 100 ng/mL. For in vivo applications, encapsulation of BMP-2 and BMP-7 in a drug delivery system can provide local, clinically relevant doses for short or long time. This is often accomplished by encapsulating BMPs in a delivery vehicle such as a biocompatible polymer microsphere or hydrogel. Controlled release of recombinant human BMP-7 in a microsphere delivery system enhanced a bone tissue engineering scaffold in a subcutaneous mouse model [110].

Parathyroid Hormone Delivery

Parathyroid hormone (PTH) is secreted by the parathyroid glands and can have both catabolic and anabolic effects on bone [111]. It is well-known that a continuous and sustained treatment of PTH will lead to increased bone resorption or reduction in bone mass [112]. Conversely, an intermittent or pulsatile treatment of PTH can enhance osteogenesis or bone regeneration. PTH is used as an FDA-approved treatment of osteoporosis, which requires daily injections of a sufficient dose to be effective. A novel approach to PTH delivery uses a multilayered scaffold to achieve local pulsatile release of PTH to stimulate bone regeneration. Layers of PTH are separated by layers of polyanhydride (PA) and sealed with PCL. Surface erosion of PA allows the sequential release of PTH from the scaffold in controlled doses. Using this approach, a daily spike in PTH was observed and corresponded to superior healing of a critical-size defect in a calvarial mouse model [113] (Fig. 48.2).

Nucleotide Delivery and Gene Therapy

Nucleic acids (DNA, RNA, small interfering RNA, and microRNA [miRNA]) regulate gene and protein expression in cells. There are several methods to enhance or suppress the expression of specific proteins or genes that are involved in healing or new tissue formation.

The goal of gene therapy is to increase or sustain the local expression of factors related to healing, cell recruitment, cell proliferation, or cell differentiation. This is most frequently accomplished by delivering nonintegrating plasmid DNA or inserting additional copies of the desired genes into the chromosomal DNA. Transfer of genes to cells at the site of injury can be accomplished by viral and nonviral delivery methods [8,114]. Transcription and translation of these additional gene copies can enhance the expression of factors that promote healing.

For bone regeneration, gene therapy often involves modulating the temporal and spatial expression of BMPs [115,116]. The use of adenovirus vectors allows researchers to transfect progenitor or differentiated cells with additional copies of genes that enhance regenerative processes. In one example, adenoviral gene transfer was used to elicit the expression of BMP-7 from muscle cells. These cells were then implanted into femoral defects to evaluate their regenerative potential [117]. Although gene transfer can be effective, improvements are needed in the efficiency, stability, and safety of these methods.

In a nonviral approach, a gene vector that incorporates a plasmid encoding for BMP-2 is protected by a poly(D,L-lactide) (PDLLA) copolymer and delivered to a mandibular defect [118]. In this strategy, the gene vector is delivered on a PDLLA scaffold to induce cells localized at the site of injury to express BMP-2 and improve regeneration of the bone defect. In a similar approach, a BMP-7 plasmid enhanced by a heparin-binding site is transfected into MSCs and applied to regenerate bone defects [119].

Local delivery of nucleic acid molecules is another promising novel approach to bone regeneration. In particular, applications that use miRNA are being developed to enhance pro-osteogenic factors or suppress antiosteogenic factors [120]. Controlled release of miRNA-26a demonstrated a significant regenerative capability in an in vivo osteoporotic mouse model. In this example, miRNA-26a increased bone regeneration and healing by targeting a known inhibitor of osteoblastic activity, glycogen synthase kinase-3β [121]. Polymer−miRNA complexes (polyplexes) were

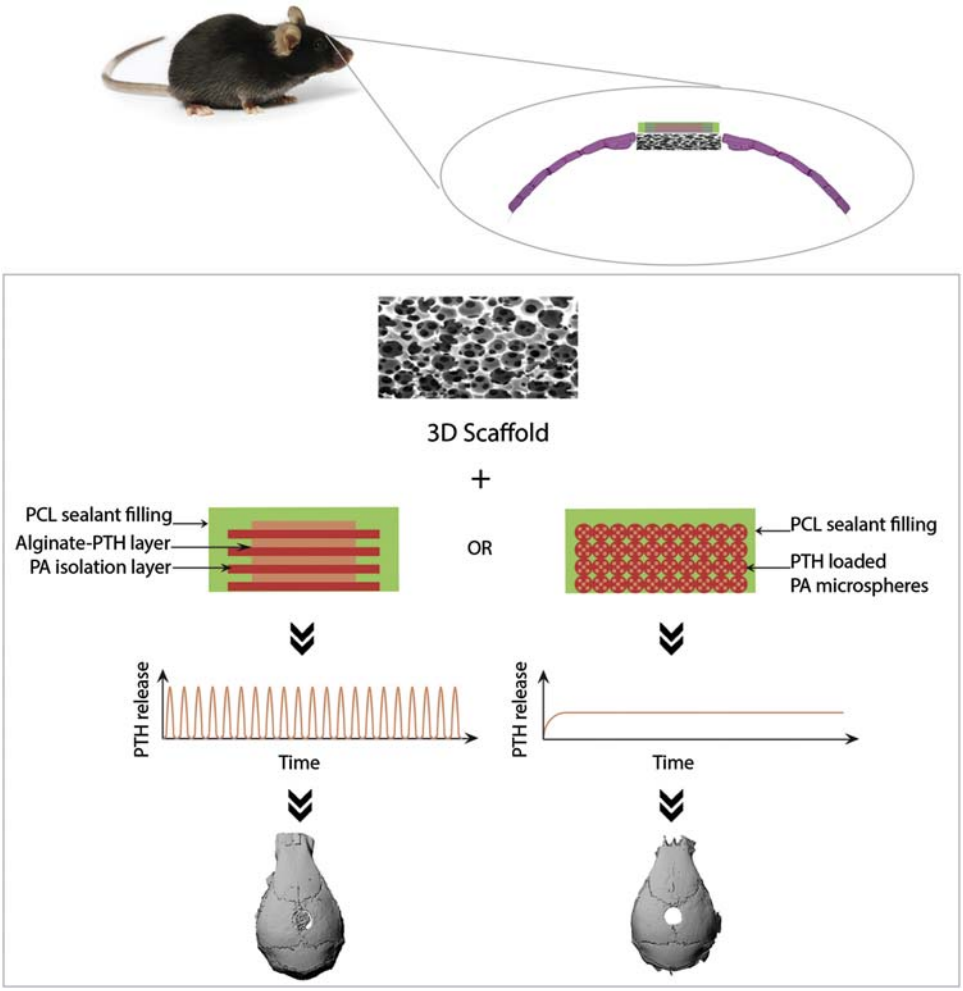

FIGURE 48.2 Experimental design of using a three-dimensional (3D) cell-free scaffold and a parathyroid hormone (PTH) deliver device (pulsatile or continuous) to repair calvarial bone defect in a mouse model. *PA*, polyanhydride.

created by mixing miRNA-26a and hyperbranched polyesters with affinity to miRNA-26a. These polyplexes were encapsulated in PLGA microspheres and were subsequently immobilized on a nanofibrous PLLA scaffold. The polyplex–microsphere–scaffold platform was surgically implanted in critical-size calvarial defects in osteoporotic mice. Efficient intracellular delivery of the miRNA was accomplished in a stepwise manner. As the PLGA microspheres were hydrolyzed, the miRNA-26a–containing polyplexes were released from their interior. These polyplexes were subsequently engulfed by cells via endocytosis. Endosomal escape allowed release of the miRNA into the cytoplasm of the cell. Fig. 48.3 Intracellular release of miRNA-26a in osteoblasts increased bone mass and increased expression of early and late genetic markers of osteogenic differentiation [121].

The advent of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-9–associated (Cas9) gene editing technology has increased the efficiency by which researchers can manipulate the genome of mammalian cells and establish new cell lines for use in culture. Although CRISPR/Cas9 has increased the ability of researchers to study bone function and regeneration [122–124], no CRISPR-based therapeutic approaches have been developed to date.

IMMUNOMODULATION IN BONE REGENERATION

During the natural process of healing of a bone injury, the inflammatory and immune response activates several distinct cell types that can directly or indirectly interact with progenitor cells, osteoblasts, and osteoclasts [125–127]. Methods to modulate these interactions are being developed to enhance bone regeneration therapies.

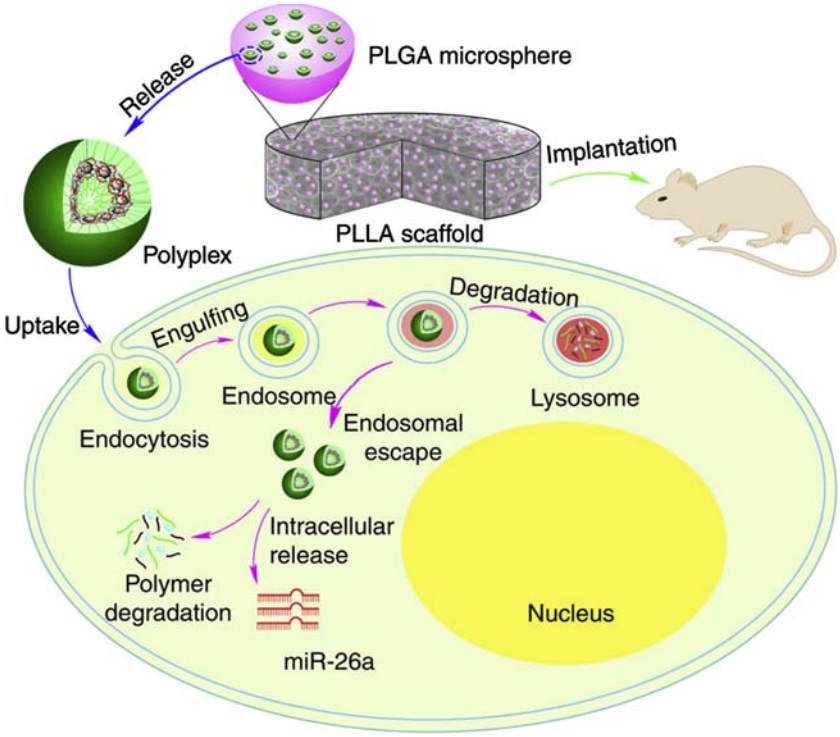

FIGURE 48.3 Hyperbranched polymers (HPs) and microRNA (miRNA) formed polyplexes in water. The HP—miRNA polyplexes were encapsulated into the poly(lactic-*co*-glycolic acid) (PLGA microspheres) via the double-emulsion method, and the PLGA microspheres containing the HP—miRNA polyplexes were then attached on the poly-L-lactic acid (PLLA) nanofibrous scaffold. The PLGA microsphere-incorporated PLLA scaffolds were implanted into mice. The HP—miRNA polyplexes released from the PLGA microspheres (on the PLLA scaffold) could be taken into cells through endocytosis. Intracellular release of miRNA in the cytosol after enzymatic polymer degradation allows its regulation of gene expression.

Macrophages

Macrophages are part of the innate immune response to injury. As they are recruited to the site of injury, macrophages act by phagocytizing bacteria and damaged tissue. They also release cytokines and growth factors that initiate and promote healing. One study demonstrated that coculture of MSCs with specific macrophage subtypes enhanced osteogenic differentiation of MSCs. Antiinflammatory macrophages (M2) promoted osteogenic differentiation of MSCs better than proinflammatory macrophages (M1) or immature macrophages (M0) [128]. In another study, M1 macrophages increased osteogenic differentiation of MSCs more dramatically than M2 macrophages [129]. These two studies are seemingly contradictory, although in both studies, coculture of MSCs with M1 and M2 macrophages enhanced osteogenic differentiation, but the importance of macrophage polarity [130] and the molecular signaling pathways involved have yet to be clearly elucidated.

In practice, macrophages have been stimulated by the release of a macrophage-recruiting agent and platelet-rich plasma from a gelatin hydrogel scaffold to a defect site in the ulna bone in a rat model demonstrated enhanced bone healing [131]. Mechanistic studies that clarify the role of macrophages in bone healing are still limited.

T Cells

The adaptive immune response also has a role in bone regeneration in the form of activated T cells. T lymphocytes regulate callus formation in bone healing by secretion of pro-osteogenic cytokines (tumor necrosis factor-α and interleukins [IL]-6 and -1β). Coculture of activated T cells with MSCs enhances osteogenic differentiation owing to the osteogenic effect of soluble factors released by $CD4^+$ T lymphocytes [132]. Furthermore, there is a differential effect on osteoblast maturation from pro inflammatory and antiinflammatory $CD4^+$ T-cell subsets. $CD4^+$ T-helper 17—specific cytokines (IL-17A and IL-17F) increased ALP activity in MSCs, and IL-17A in particular works synergistically with BMP-2 to increase calcium deposition by MSCs.

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CHAPTER

49

Hair Cell Regeneration in the Inner Ear and Lateral Line

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INTRODUCTION

The ability to detect sounds and motion is mediated through mechanosensory hair cells located in the inner ears of all vertebrates. The basic cellular structures of hair cells are similar across vertebrate classes, but mammals are unique in their inability to regenerate hair cells that have been lost as a result of aging or trauma. As a result, mammals are the only vertebrates that incur lasting deficits in hearing or balance. In this chapter, we will describe the process of hair cell regeneration that occurs in nonmammalian species, explore the factors that prevent regeneration in mammal inner ears, and discuss efforts to induce regeneration using transgenic mouse models and gene therapy.

STRUCTURE OF THE INNER EAR

The inner ear can be grossly divided into three structures: the membranous labyrinth, a series of epithelial-lined fluid filled chambers derived from the otocyst; the bony labyrinth, a dense bony structure that surrounds and protects the membranous labyrinth; and the eighth cranial nerve, also called the vestibulocochlear nerve, which provides afferent innervation to all sensory structures within the inner ear (Fig. 49.1). The membranous labyrinth can be further subdivided into a ventral auditory section that contains the cochlea and a dorsal vestibular section that contains the three semicircular canals that act to mediate balance, as well as two otolithic organs, the saccule and utricle, which have a role in perception of linear acceleration and gravitation forces. Each of these structures contains a sensory epithelium that is composed of mechanosensory hair cells and a population of surrounding epithelial cells referred to as supporting cells. Vestibular sensory epithelia appear as rounded or oblong patches containing thousands of mechanosensory hair cells. The overall structure of these sensory patches is grossly similar among all vertebrate classes. In contrast, auditory epithelia show a greater degree of diversity; some develop as narrower, elongated stripes of cells. The most extreme example of this is the mammalian auditory sensory epithelia, also called the organ of Corti, which features four or five rows of hair cells extending up to 70 mm [1], which are functionally and morphologically divided into one row of inner hair cells that detect auditory stimuli and three or four rows of outer hair cells that serve as a mechanical amplifier. Finally, aquatic vertebrates such as fishes and amphibians have an additional hair cell-based sensory system called the lateral line, which is composed of a series of canals and sensory patches arrayed along the outer surface of the head and body, and which enables the detection of flow and vibrations in the surrounding water.

Mechanosensory hair cells are so named because of the presence of a modified bundle of microvilli, referred to as stereocilia, that project from the lumenal surface of each cell [2,3]. The morphology of the bundle is characterized by an asymmetric staircase pattern in which the lengths of the individual stereocilia increase toward one end of the bundle. Deflection of the bundle in the direction of the longest stereocilia leads to opening of transduction channels, the influx of positively charged ions, and subsequent increases in the rate of neurotransmitter release. Each hair cell

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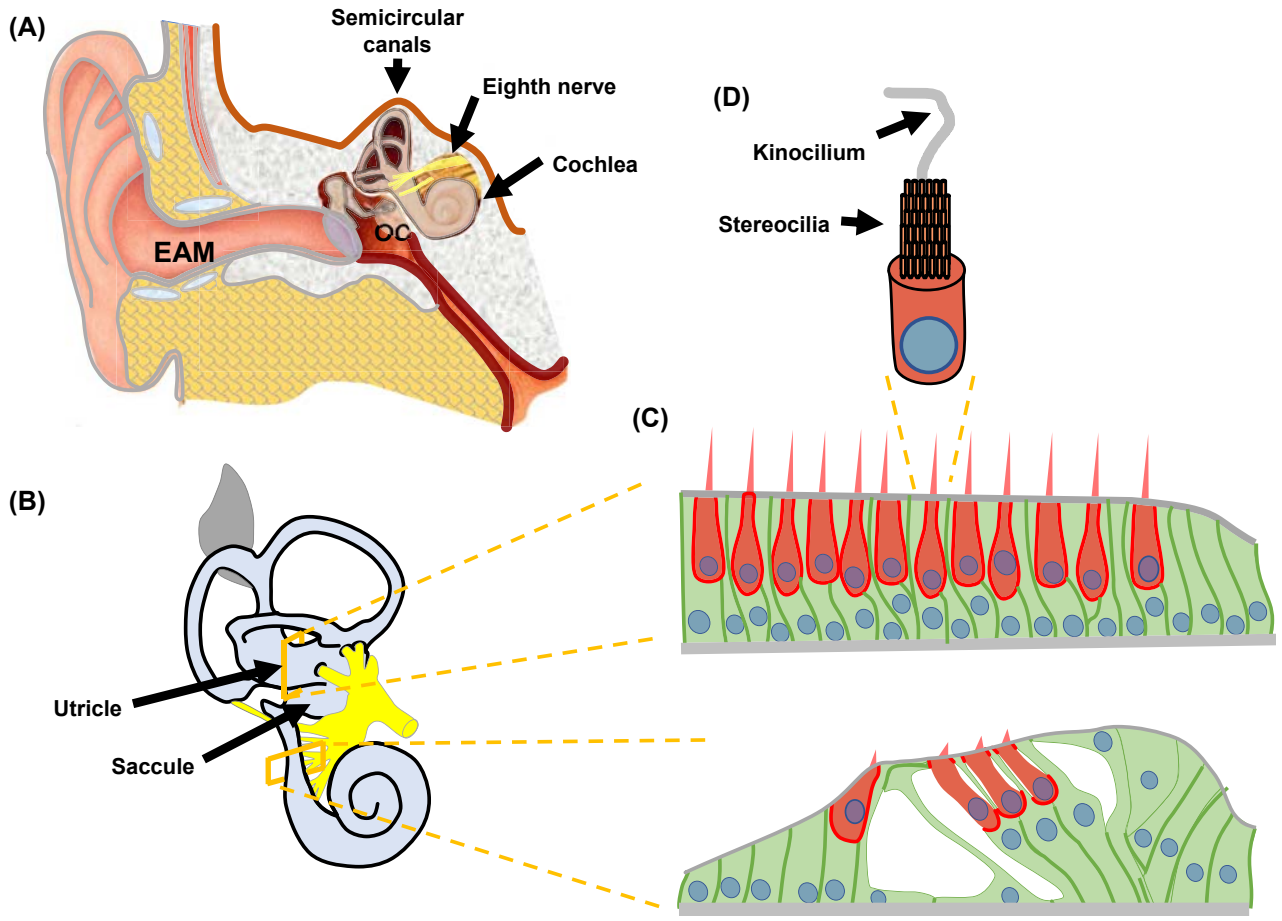

FIGURE 49.1 Overview of the inner ear. (A) Cut away view of the human ear. The pinna and external auditory meatus (EAM) transmit sound waves to the tympanic membrane located at the central end of the EAM. Vibration of the tympanic membrane is amplified through the middle ear ossicles (OC). The footplate of the stapes vibrates the footplate of the stapes, which creates a pressure wave that travels along the cochlear spiral. The three semicircular canals, along with the utricle and saccule, mediate sense of balance, acceleration, and gravity. Signals generated in the inner ear are carried to the central nervous system through separate branches of the eighth nerve. (B) View of the inner ear illustrating the three semicircular canals, cochlear spiral, and endolymphatic duct (*dark gray*). The branches of the eighth nerve are illustrated in *yellow*. (C) Schematic cross-sections of the sensory epithelia in the utricle (*upper image*) and cochlea (*lower image*). Hair cells are *red* and supporting cells are *green*. In the utricle, hair cells are arranged in a large patch whereas in the cochlea, hair cells are arranged in four or five rows that extend along the length of the cochlear spiral. (D) Schematic of a single utricular hair cell illustrating the staircase pattern of the stereocilia (*orange*) and the single kinocilium (*gray*).

forms synapses with peripheral axons from one or more afferent neurons that transmit changes in the rate of neurotransmitter release into brain stem nuclei and higher central nervous system structures, where these signals are interpreted as perceptions of sound, vibration, or movement.

HAIR CELL LOSS

A number of factors have a role in the death of hair cells. Foremost among these is aging. According to the National Institute on Deafness and Other Communication Disorders, nearly 35% of all individuals will have a significant hearing loss by age 65 years, and this number will increase to over 50% by age 80 years. Similarly, virtually all individuals lose the ability to perceive frequencies above 17 kHz by age 25 years. The underlying cellular or genetic mechanisms that lead to this progressive hair cell loss remain largely unknown, although mutations in some genes, in particular *Cdh23*, have been shown to cause age-related hearing loss in mice [4]. Similar declines in hair cell numbers have also been reported in aged vestibular epithelia; however, a strict correlation between hair cell loss and loss of vestibular function has not been established [5].

Environmental factors can significantly accelerate the rate of hair cell loss. Exposure to loud noises, even in the short term, can lead to hair cell death from both mechanical damage and neurotoxicity from calcium, resulting in hearing loss. Toxins such as copper and *Gingko biloba* have also been shown to be harmful to hair cells [6,7]. In addition, several medicines, most notably aminoglycoside antibiotics and the anticancer drug cisplatin, have been shown to kill hair cells [8]. As is the case for age-related hearing loss, the cellular basis for hair cell death after exposure to any of these stressors is not particularly well-understood, but metabolic stress and generation of reactive oxygen species, may be important mediators [9]. This is particularly true for outer hair cells in the organ of Corti, which are highly sensitive to all environmental stressors. Outer hair cells are motile cells that generate cellular contractions in response to stimulation. Because these contractions typically occur at the optimal frequency for each cell, some cells may need to contract repeatedly at rates as high as 20 kHz. This high level of activity may produce a chronic metabolic stress that makes the outer hair cells particularly sensitive to additional external challenges.

HISTORY OF HAIR CELL REGENERATION

As discussed in the Introduction, in mammals, hair cells are primarily generated during the embryonic or early postnatal period, depending on the species. Therefore, in adults, hair cell loss is permanent. Before the 1980s, it was assumed that similar limitations existed in the inner ears of all other vertebrates. However, examinations of the inner ears of sharks and rays, which grow indeterminately, indicated an ongoing increase in the number of hair cells in the inner ear sensory patches of these animals. In several species of sharks, the increase in the number of hair cells is remarkable; more than 180,000 cells are added to just a single inner ear sensory patch, the macula neglecta, over the life of an individual animal [10]. Subsequent studies in amphibians used a mitotic tracer, tritiated-thymidine, to demonstrate that new hair cells in the inner ears of these animals were generated through cellular proliferation of surrounding cells [11]. These results motivated additional studies using chickens, homothermic vertebrates with determinant growth, and an elongated auditory structure, the basilar papilla (BP), which is functionally similar to the mammalian cochlea. After induction of hair cell damage using prolonged exposure to loud pure tones, initial experiments used scanning electron microscopy to image the BP [12]. Whereas BPs imaged immediately after noise exposure showed widespread hair cell loss and damage, analysis of similar BPs after a 2- to 4-week recovery period illustrated nearly complete recovery of the hair cell population. Moreover, recovery of auditory function tracked closely with morphological recovery, demonstrating that the new hair cells were functional and that the rest of the auditory system remained intact after exposure to noise. Finally, introduction of a mitotic tracer demonstrated that at least some of the regenerated hair cells arose from proliferation of the surrounding supporting cells [13,14]. To determine whether this regenerative ability is restricted to young birds, similar experiments were performed in aged quails [15]. Even quails that were near the end of their expected life span (3 years) were able to regenerate hair cells and recovery auditory function, which demonstrated that this ability is retained throughout the life of the animal.

Subsequent studies at the cellular level identified two different mechanism for the formation of regenerated hair cells (Fig. 49.2). As discussed previously, some new hair cells arise from the re-entry and subsequent division of supporting cells, but in other cases, supporting cells are able to convert into hair cells directly, a process referred to as transdifferentiation [16,17]. These results demonstrate that in birds, supporting cells can act as hair cell progenitors. This suggests that these cells retain some stem or progenitor capacity throughout the life of the animal. Whether every supporting cell can act as a stem or progenitor cell or whether this ability is limited to a subset of cells within the epithelium has not been determined. Interestingly, the response of supporting cells, in terms of whether they transdifferentiate or reenter the cell cycle, is spatially segregated; cells located on one edge of the BP are more likely to transdifferentiate whereas those on the other edge are more likely to re-enter the cell cycle [18]. The molecular and/or functional bases for these differences have not been determined.

A final consideration in the avian system is the unexpected observation that vestibular epithelia undergo constant turnover of hair cells; old cells are replaced with new ones such that the average life of a hair cell in a vestibular sensory epithelium is approximately 1 month [19]. This process may be a compromise of the ancestral trait of ongoing addition of hair cells, as seen in fish and amphibians, with the derived trait of determinant growth as occurs in birds and mammals. However, this also demonstrates that vestibular epithelia can efficiently maintain all of the neuronal connections required for normal function even as hair cells are being continually lost and regenerated.

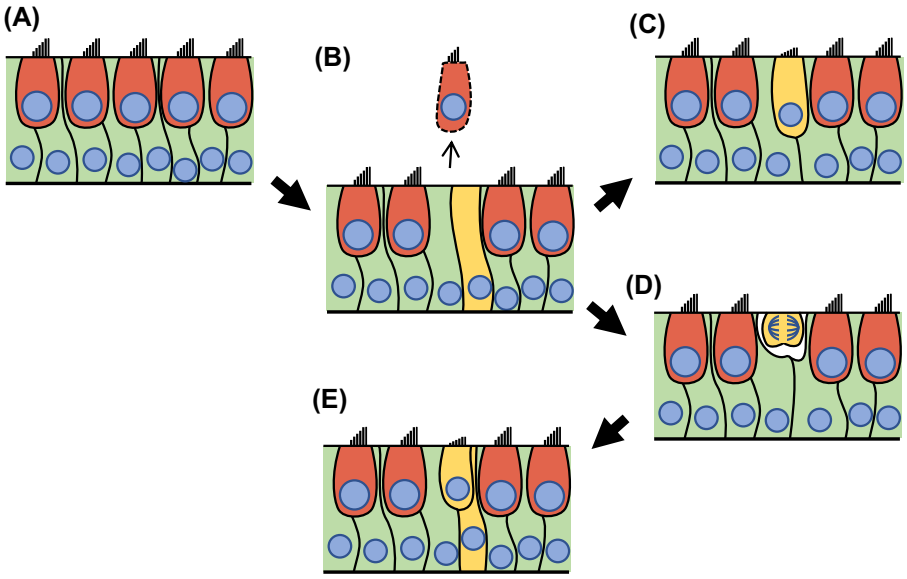

FIGURE 49.2 Modes of hair cell regeneration in the chick basilar papilla. (A) Cross-sectional view of an idealized basilar papilla. Hair cells (*red*) and supporting cells (*green*) are present in a pseudostratified epithelium. (B) In response to an insult, hair cells are ejected from the epithelium (*small arrow*), creating a gap in the mosaic of hair cells and supporting cells and eliciting a response from one or more of the remaining supporting cells (*yellow*). (C) Transdifferentiation. Under some circumstances one of the remaining supporting cells will directly transform into new hair cells (*yellow*). This results in a decrease in the number of supporting cells. (D) Proliferative regeneration. The second possible response is for a remaining supporting cell (*yellow*) to undergo mitotic proliferation to generate new progenitor cells. (E) These cells then go on to form new hair cells and supporting cells (*yellow*).

SPONTANEOUS HAIR CELL REGENERATION IN MAMMALIAN VESTIBULAR ORGANS

The discovery of spontaneous hair cell regeneration in birds sparked a re-examination of the regenerative potential within adult mammalian vestibular and auditory epithelia. As a first step, work from Warchol et al. [20] and Forge et al. [21] demonstrated limited proliferation of supporting cells in cultured utricles from Guinea pigs and humans, as well as the presence of hair cells with bundles that appeared immature in Guinea pigs. Although these results were compelling, the number of potential new cells was small and a preponderance of clinical data clearly suggests that meaningful vestibular hair cell regeneration does not occur in adult humans. In contrast to the findings in the utricle, similar studies in the adult mammalian auditory system confirmed the absence of hair cell regeneration in this epithelium [22]. Despite the importance of the findings in the utricle, further examination of potential hair cell regeneration in this structure was slowed by difficulty in reliably killing hair cells in the vestibular system in vivo.

In the auditory system, two different approaches have been developed to kill hair cells reliably and reproducibly: exposure to loud sounds and administration of aminoglycoside antibiotics such as neomycin or kanamycin, alone or combined with a loop-diuretic such as furosemide [23]. The effects of both of these treatments have been studied extensively and have led to precise protocols that yield consistent results. Moreover, because the auditory system is organized along a tonotopic gradient, damage can be mapped to particular regions of the auditory epithelium based on the results of tests for auditory sensitivity at different frequencies [24]. Because vestibular epithelia are not stimulated by auditory vibrations, exposure to loud sound does not cause hair cell loss, and whereas the sensitivity of vestibular hair cells to aminoglycosides is comparable to auditory hair cells in vitro, systemic treatments result in variable hair cell death. Moreover, many animals that exhibit significant vestibular loss of function after a chemical insult will show progressive and marked recovery over time as a result of functional compensation based on visual input [25]. As a result, between the late 1980s and the early 21st century, it was virtually impossible to assess the extent of hair cell regeneration accurately in vestibular epithelia in vivo because there was no way to kill the existing hair cells consistently. However, the situation changed with the development of several lines of transgenic mice beginning around 2010. First, lines were developed in which genes that are expressed specifically in supporting cells drive the expression of cre recombinase (cre). When one of these lines is combined with one of several reporter lines in which expression of cre leads to the permanent expression of a fluorescent molecule such as green fluorescent protein (GFP), it is possible to differentiate regenerated hair cells that will express GFP because they arise from supporting cells, from nonregenerated hair cells that are GFP-negative [26]. In addition, in 2013, Rubel and colleagues [27] developed a mouse model in which the promoter

for the hair cell–specific gene *Pou4f3* is used to drive expression of the human diphtheria toxin receptor (Dtr). Because the human Dtr is approximately 10,000 times more sensitive to diphtheria toxin [28] compared with the mouse Dtr, this line can be used to kill vestibular (and auditory) hair cells effectively and consistently by giving mice injections of diphtheria toxin.

In fact, injection of diphtheria toxin in this line consistently kills approximately 94% of hair cells in the utricle by 14 days after treatment [27]. Animals killed at specific recovery times between 15 and 180 days showed a modest recovery of hair cells, up to approximately 17% of the original number, and the loss of supporting cells and lack of incorporation of mitotic markers demonstrated that these cells arise from the conversion of surrounding supporting cells into hair cells. In addition, a small percentage of regenerated hair cells arose as a result of mitotic division; the number of mitotically generated cells decreased rapidly as animals aged past the very early postnatal period [27,29]. These results provide definitive evidence that a limited amount of spontaneous hair cell regeneration can occur in adult mammalian vestibular epithelia. However, whether the regenerated hair cells lead to recovery of function remains to be determined.

ROAD BLOCKS TO REGENERATION

An intriguing question which, if answered, might provide insights regarding the development of clinical strategies, is: Why do mammals have a greatly reduced capacity to regenerate hair cells, including a complete loss in the auditory system? At a systems level, one of the most appealing hypotheses is that the increased complexity of the organ of Corti relative to other auditory epithelia, and in particular the highly differentiated state of the supporting cells within the organ of Corti, has resulted in those cells losing the ability to de-differentiate, as might be required to change fate or re-enter the cell cycle. Although the basis for such a loss is not clear, one possibility would be alterations in the epigenetic landscape of the supporting cells, leading to an inability to reactivate important genes required for one or more aspects of regeneration [30]. There is ample evidence from other systems that is consistent with a negative correlation between cellular differentiation and plasticity, sometimes correlated with epigenetic changes, which supports this idea [31]. However, a noteworthy caveat is that this hypothesis does not explain why regenerative ability would also be greatly decreased in the vestibular system where supporting cells appear to be considerably less differentiated compared with similar cells in the organ of Corti and are comparable to those in nonmammalian vestibular organs. Still, it is possible that changes in the structural, transcriptional, or epigenetic state of auditory supporting cells might also lead to similar changes in other inner ear supporting cells.

The reduced capacity for mammalian supporting cells to regenerate hair cells seems to be tied to their own maturation. Generally, loss of cochlear or vestibular hair cells during embryonic or perinatal periods results in supporting cell proliferation and hair cell regeneration [29,32], although the capacity for regeneration decreased rapidly with postnatal age. Corwin and colleagues provided intriguing, albeit correlative, data suggesting that a structural component of supporting cells, dense actin belts located just beneath the lumenal surfaces of supporting cells, may act to inhibit the ability of those cells to undergo a regenerative response [33,34]. At early postnatal time points, lumenal cortical actin in supporting cells creates a thin circumferential belt located close to the lateral cell membrane. As discussed, supporting cells in the utricle generate a significant regenerative response, including cellular proliferation, during this same period. However, as an animal ages, the width of supporting cell lumenal actin belts increases while regenerative ability decreases. Examination of actin belts in non-mammalian vertebrates, including birds and fish, revealed thin belts similar to those observed in newborn mammals, regardless of the age of the animal. Unfortunately, it has not yet been possible to disrupt these belts to demonstrate whether they actually prevent supporting cells from initiating a regenerative response. The ability of postnatal supporting cells to respond to growth factors and extracellular matrix components in culture also decreases rapidly [35,36]. Thus, the lack of mammalian regeneration appears to be a trait acquired by the maturation of supporting cells.

INSIGHTS FROM DEVELOPMENTAL BIOLOGY

Because meaningful spontaneous regeneration does not occur in mammals, it seems likely that manipulation of the system, possibly through pharmacological and/or genetic approaches, will be necessary to induce a response. To develop an appropriate strategy, it will be necessary to identify the molecular and genetic pathways that regulate the key steps in a regenerative response. Based on the process that occurs during hair cell regeneration in non-mammalian vertebrates, hair cell regeneration can come from the nonmitotic conversion of supporting cells into

hair cells or the proliferation of supporting cells followed by differentiation of some of the progeny into hair cells. Because neither proliferation of supporting cells nor differentiation into hair cells occurs at a high rate in adult mammalian hair cell epithelia, one possible way to identify the factors that regulate each of these events is to examine them during development.

Significant progress has been made in understanding both proliferation and differentiation, but our understanding of the regulation of differentiation is more advanced and therefore will be discussed first. One of the initial findings was actually related to a genetic signal that inhibits rather than promotes hair cell formation (Fig. 49.3). The Notch signaling pathway is an ancient developmental process that regulates the number of cells that assume a particular cell fate through cell-cell based lateral inhibition [37]. Briefly, transmembrane Notch receptors are activated through binding similarly membrane-bound ligands called Deltas or Jaggeds. Because both ligands and receptors are membrane bound, cell−cell contact is required for receptor activation. After binding, Notch molecules are cleaved to generate a Notch intracellular domain (NICD) molecule that is then translocated to the nucleus of the cell, where it forms a transcriptional complex with other factors including Rbpj. The results of Notch activation vary, depending on the biological system, but a common effect is the inhibition of cellular differentiation through the induction of expression of a class of inhibitory transcription factors that includes Hes1, Hes5, and Hey2. In the developing cochlea, Notch1 is expressed broadly in the progenitor cells that will develop as both hair cells and supporting cells [38,39]. As cells begin to develop as hair cells, they upregulate expression of two Notch ligands, Jagged2 and Delta1. Binding of these ligands leads to the generation of NICDs and expression of Hes genes in surrounding cells [38,40,41]. Deletion of any component of this signaling pathway or prevention of Notch cleavage by pharmacological γ-secretase inhibitors results in an increase in the number of cells that develop as hair cells, which demonstrates that the Notch signaling pathway acts as an inhibitor of hair cell development [38,42]. Whether this pathway is still active in the adult auditory epithelium and could therefore have a role in inhibiting hair cell regeneration, is a matter of debate [43−45]. However, some initial results to be discussed subsequently suggest that this is possible.

The demonstration of a role for Notch signaling also provided clues regarding some of the genes that might have a positive role in inducing hair cell formation. As discussed, Notch is part of an ancient signaling pathway that includes Hes and Hey transcription factors. These transcription factors are part of a large family that share common structural motifs including a basic DNA binding domain (b) and a helix−loop−helix (HLH) dimerization domain. Other members of the basic HLH family include molecules that promote cellular differentiation [46]. Screening for basic HLHs expressed in developing hair cells indicated that Atoh1 might be a strong candidate. Results of both in situ hybridization and mouse reporter line studies demonstrated that Atoh1 turns on in a large number of cochlear progenitor cells before hair cell development, but by embryonic day (E)16 to E17 in the mouse, expression is restricted to the developing hair cells [47,48]. More important, genetic deletion of *Atoh1* leads to a complete absence of all inner ear hair cells whereas forced expression of Atoh1 in the embryonic inner ear can induce cells to adopt a hair cell fate [47,49,50]. Finally, lineage tracing experiments demonstrated that the number of cells that initially express *Atoh1* is greater than the number that ultimately develop as hair cells and that activation of Notch signaling has a critical role in determining which cells will maintain expression of Atoh1 [51]. Based on these results, Atoh1 has been established as a strong inducer of hair cell formation.

Following from these experiments, both expression of Atoh1 and modulation of Notch signaling have been examined as possible mechanisms to induce hair cell regeneration in the mature inner ear. Although promising in some respects, the results have unfortunately, been limited. To assess the ability of cells in different regions of the inner ear to develop as hair cells, transgenic mice in which broad expression of Atoh1 can be induced pharmacologically were generated by two laboratories. The results indicated that whereas forced expression of Atoh1 induces ectopic hair cell formation in embryonic or neonatal cochlea, this ability is completely lost in adult inner ear cells [52,53]. Furthermore, even in neonatal tissue, hair cells that were formed in response to forced expression of Atoh1 failed to mature completely. The reasons for both lack of hair cell maturation and loss of responsiveness to Atoh1 are unclear. The most likely explanation for the defects in hair cell maturation are that other factors (either co-factors for Atoh1 or possibly additional transcription factors) are required to drive hair cell maturation. This hypothesis was supported by a study showing that the efficacy and maturity of hair cells derived from embryonic stem cells could be significantly increased if the cells were forced to express three relatively inner ear−specific transcription factors: Atoh1, Gfi1, and Pou4f3 [54]. Gfi1 and Pou4f3 are expressed in hair cells soon after Atoh1, but both loss-of-function and gain-of-function experiments indicated that neither Gfi1 nor Pou4f3 is necessary or sufficient for initial hair cell formation [55,56]. Regardless, these results suggest that the combination of these three factors may have the ability to induce more mature hair cells in an adult inner ear epithelium, although this possibility has not yet been directly tested.

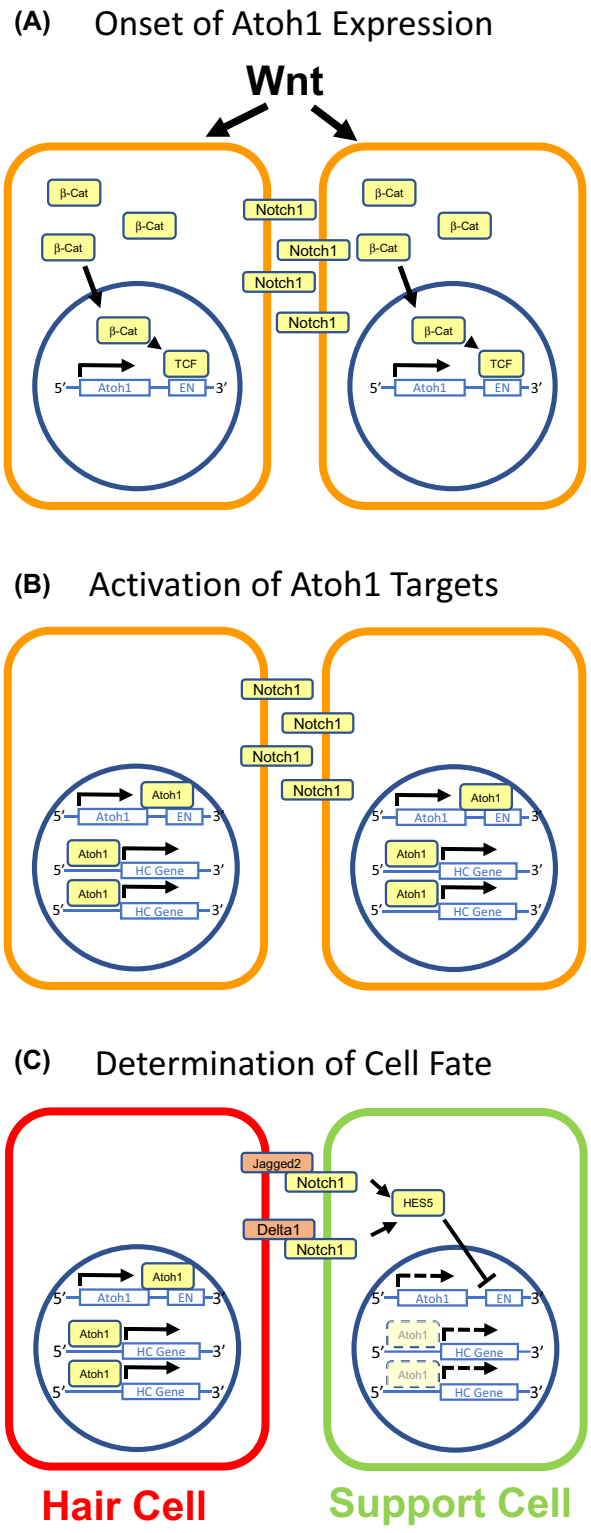

FIGURE 49.3 Regulation of cell fates during inner ear development. Three stages of molecular signaling interactions are illustrated. (A) An initial period of Wnt signaling leads to stabilization and subsequent nuclear-translocation of β-catenin (β-cat). In the nucleus, β-catenin induces expression of TCF/Lef transcription factors that bind directly to an *Atoh1*-3′ enhancer leading to the initial expression of *Atoh1*. (B) Atoh1 protein binds to multiple DNA recognition sites, including one in the Atoh1-3′ enhancer and others in uncharacterized hair cell (HC) genes. (C) In some cells, expression of two Notch ligands, Delta1 and Jagged2, is initiated. Delta1 and Jagged2 bind to Notch1 receptors on neighboring cells, causing the upregulation of inhibitory transcription factors such as HES5. HES5 inhibits the expression of Atoh1, resulting in a downregulation of Atoh1 target genes, including *Atoh1*. Cells that maintain expression of Atoh1 go on to develop as hair cells whereas cells in which Atoh1 is down-regulated develop as supporting cells.

Regarding the loss of ability of Atoh1 to induce hair cells in more mature inner ear cells, the most likely explanation for this change is progressive epigenetic changes that prevent Atoh1 from binding to and activating essential downstream target genes. Although this type of epigenetic modulation has been reported in other cell types, no definitive studies have been performed in the inner ear. Other possibilities include downregulation of essential co-factors or required post-translational modifications that do not occur in more mature cells.

The results described earlier focused on the cochlear epithelium; however, an experiment demonstrated consistent, although not entirely similar, results for the vestibular epithelia. As is true for the cochlea, forced expression of Atoh1 induced hair cell formation in utricular epithelia at early postnatal ages and the efficacy of this effect decreased over time with 75% fewer hair cells formed in response to Atoh1 expression at postnatal day 21 (P21) versus the same level of induction at P0 [57]. However, the formation of new hair cells in the utricle at P21 contrasted with results from the cochlea in which no new hair cells were formed at that age. Unfortunately, the ability of Atoh1 to induce new hair cells in the utricle was not tested at ages older than P21, so it is not clear whether the level of induction observed at P21 represents a plateau or whether further loss of efficiency would occur at older ages. Similar experiments in which Atoh1 expression was induced using viral vectors in more mature animals reported the formation of a limited number of hair cell–like cells in different regions of the inner ear, which is consistent with the possibility that some adult inner ear cells located outside the cochlear duct retain the ability to develop as hair cells [58]. In fact, recovery of some degree of vestibular function was reported in animals in which hair cells were killed using systemic applications of aminoglycosides and loop diuretics [23]. However, as discussed previously, it was difficult to determine the relative contributions of hair cell regeneration and functional compensation in those experiments.

During development, activation of the Notch pathway serves to inhibit hair cell formation. This observation raised the question of whether continued activation of this pathway, in particular in response to injury, might have a role in inhibiting hair cell regeneration. Consistent with this idea, examination of the expression of Notch pathway genes in the chicken basilar papilla indicated that both Notch and its receptors are re-expressed during a regenerative response, although these factors were subsequently downregulated once the epithelium completed the regenerative process [59]. Neither Notch1 nor the ligands Delta1 and Jagged2 were observed in the mature intact organ of Corti. However, examination of epithelia after noise trauma yielded contrasting results; one laboratory reported an increase in the expression of Notch pathway genes, whereas a second observed the opposite result [43]. More intriguing are the results of a study in which partial recovery of hearing sensitivity was demonstrated after noise trauma in animals treated with a systemic γ-secretase inhibitor [45]. γ-Secretase is required for the cleavage of Notch [60]. Although the recovery was modest, the results suggested a potential role for Notch signaling in inhibiting hair cell regeneration.

Finally, the Wnt signaling pathway has been shown to have diverse roles in hair cell development. Wnts are secreted glycoproteins that have been implicated in myriad biological processes including cell fate, cellular polarity, and the regulation of cancer [61]. Studies in which a highly conserved target of the canonical Wnt signaling pathway, β-catenin (β-cat), was modulated both in vitro and in vivo demonstrated important roles for β-cat, and presumably Wnt signaling, in cochlear proliferation and subsequent hair cell formation [62,63]. In particular, inhibition or deletion of β-cat causes a significant disruption in hair cell development whereas increased activation of β-cat leads to increased proliferation of cochlear prosensory cells and subsequent formation of extra hair cells. Unfortunately, it has been difficult to identify the specific Wnts and Wnt receptors that may mediate these effects. A study used a combination of microarray analysis and in situ hybridization to identify and profile the expression of Wnts and Wnt receptors in the developing cochlea, but because of redundant expression and function, it is not yet possible to determine which factors might act to activate Wnt signaling within the cochlea.

As discussed earlier, under some circumstances, cellular proliferation may be required to facilitate hair cell regeneration. For instance, the loss of structural differentiation that typically occurs before cellular mitosis may increase the ability of an individual cell to undergo a phenotypic switch. In some epithelia, such as the organ of Cori, the limited number and high degree of specialization of supporting cells may necessitate the generation of new cells to maintain a functional epithelium after the conversion of some supporting cells into new hair cells. Cellular proliferation is minimal to non-existent in adult mammalian inner ear sensory epithelia [64]; however, before hair cell differentiation, the progenitor cells that will develop as hair cells are highly proliferative. Understanding how cellular proliferation is promoted or inhibited in these cells could provide valuable insights regarding possible approaches to inducing mature supporting cells to reenter the cell cycle. Extensive previous studies have identified a large number of factors that modulate cell cycle progress and exit. The first of these to be examined in the developing cochlea was the cell cycle inhibitor Cdkn1b (formerly $p27^{kip1}$). Cdkn1b is specifically expressed in all cochlear prosensory cells before terminal mitosis; expression is maintained in supporting cells through

adulthood [65,66]. Deletion of *Cdkn1b* results in approximately one additional round of mitosis within the prosensory population, leading to the generation of supernumerary hair cells and supporting cells and to auditory dysfunction. Subsequent experiments demonstrated important roles for a number of other cell cycle regulators including cyclin D1, N-myc, retinoblastoma, p130, p19, and p21 [67]. Deletion of factors that promote proliferation such as N-myc leads to reduced cells within the inner ear and significant defects in cellular patterning, whereas deletions of cell cycle inhibitors leads to supernumerary cells and some level of cell cycle reentry in adults, although in most cases those cells undergo subsequent cell death.

Overall, studies on the development of the inner ear sensory epithelia have provided valuable insights regarding the genes and signaling pathways that regulate processes relevant to hair cell regeneration. In particular, identification of the transcription factor Atoh1 as a strong inducer of a hair cell fate and the demonstration that the Notch signaling pathway has a critical role in inhibiting cells from differentiating as hair cells suggest two possible approaches to enhancing hair cell regeneration in an adult epithelium. Similarly, the discovery that the Wnt signaling pathway and cell cycle regulators act to modulate proliferation of inner ear progenitor cells may provide insights regarding methods to induce cell cycle reentry in an adult inner ear. Results described in the next section will discuss initial attempts to induce regeneration by modulating these pathways.

INDUCTION OF HAIR CELL REGENERATION USING TRANSGENIC MICE

The development of inducible transgenic mouse lines in which specific genes can be activated or inactivated in specific cell types at specific time points has revolutionized the ability to study inner ear regeneration. Based on the results of the developmental studies described earlier, four genes (Cdkn1b, Atoh1, Notch1, and β-cat) have been targeted. In most cases, two general concepts were addressed individually or together. The first of these is that forced activation of Atoh1 alone or combined with the deletion of Notch1 can enhance hair cell differentiation by expressing a hair cell inducer (Atoh1) and removing a hair cell inhibitor (Notch1). The second is that removal of the cell cycle inhibitor Cdkn1b might allow supporting cells to reenter the cell cycle. Finally, activation of the Wnt pathway has been implicated in both proliferation and differentiation, and so it might be able to mediate both events. For the bulk of these projects, the basic experimental design has been to use supporting cell-specific inducible cre lines combined with floxed deletion or activator lines to modulate one or more of these factors after injury [68,69]. These approaches have yielded encouraging results after induced hair cell loss in neonatal cochleae; unfortunately, similar results have not been obtained in adult inner ears. Because newborn mouse pups do not begin to hear until approximately 14 days postpartum, it seems possible that the results obtained in neonates may be a remnant of the embryonic developmental program or immaturity of the supporting cells. Whether the inability of these factors to induce new hair cells in adult cochleae is a result of changes in post-transcriptional or post-translational processing, loss of obligate co-receptors, epigenetic changes, or a progressive loss of stem cells within the epithelium remains to be determined.

The results of two studies shed some light but also provide some confusion regarding the mechanism that might act to prevent hair cell regeneration in the cochlea. As discussed, activation of Notch1 requires a γ-secretase–dependent cleavage of the Notch1 protein leading to the formation of NICD, which is then translocated to the nucleus to initiate signaling. Several pharmacological inhibitors of γ-secretase have been shown to block Notch1 activation effectively in vitro. Mizutari and colleagues induced hair cell damage in adult mice using noise or driving caspase expression in hair cells to promote apoptosis and then injected LY411575, a new, highly potent γ-secretase inhibitor into the inner ears of these animals for several days [45]. The mice in these studies also carried a lineage marker that allowed the authors to mark all of the supporting cells in the cochlea permanently. After 3 months of recovery, the LY411575-treated animals showed some improvement in auditory function and evidence of replacement hair cells in the cochlea. Moreover, the new hair cells expressed the lineage marker, which suggested that they had developed from existing supporting cells, although it is not clear whether they were exclusively from nonmitotic conversion or whether any proliferative regeneration was induced. This result was particularly exciting in that the use of a pharmacological agent is well-suited for development as a clinical application. From a biological standpoint, this result suggested that the Notch pathway remains active or is re-activated after injury in adult tissue. In fact, both this study and work from a separate laboratory indicated re-expression of some components of the Notch pathway in response to injury [44].

However, a subsequent study by a different group of researchers observed a different result. In that case, explant cultures of neonatal cochleae were established, treated with γ-secretase inhibitors and then assayed for the development of new hair cells. The results indicated a marked decrease in the number of new hair

cells that developed even in cochleae as young as P3. In addition, polymerase chain reaction analysis of the expression of Notch pathway genes after noise damage in adult animals indicated no reactivation of the Notch pathway [43]. Thus, these two studies would seem to present two highly disparate results for which the bases for the differences cannot easily be determined. One possibility is that the mechanisms of hair cell damage were different, as were the time scales. Another possibility is that although Notch genes are a main target of γ-secretase, they are not the only target, which raises the possibility that the regeneration observed in vivo is mediated through a different pathway. Therefore, although the results are intriguing, additional studies are clearly required.

STUDIES OF HAIR CELL REGENERATION USING THE LATERAL LINE

As discussed, in mammals and other terrestrial vertebrates, hair cell sensory epithelia are confined to the inner ear. In contrast, aquatic vertebrates, such as fishes and amphibians, possess an additional hair cell sensory structure, the lateral line system (Fig. 49.4). This sensory system is composed of a series of organs, termed neuromasts, arrayed along the surface of the skin or in bony dermal canals that connect to the skin surface (Fig. 49.4A). The amphibian lateral line is indeed the first place that hair cell regeneration was observed. After amputation and subsequent

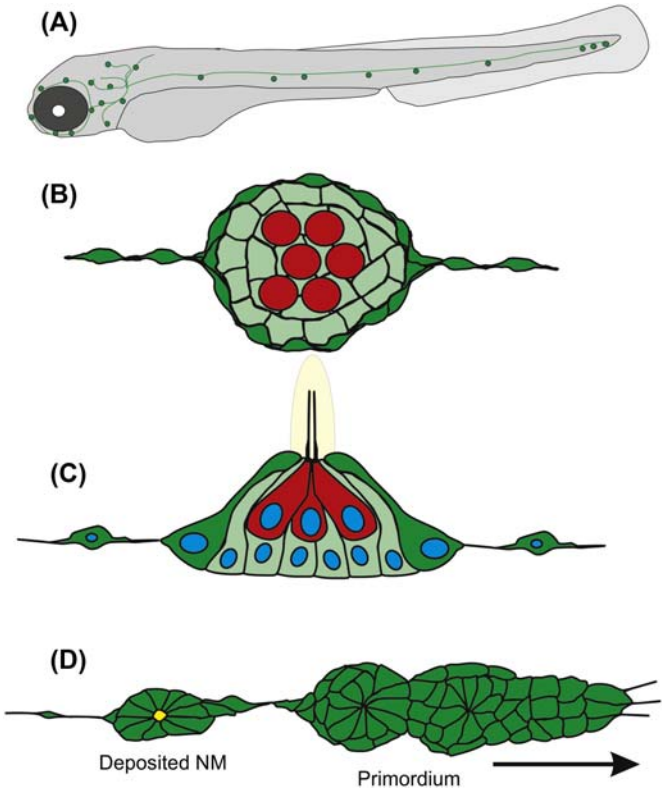

FIGURE 49.4 Overview of the lateral line. (A) Schematic diagram of the lateral line in a 4-day-old zebrafish embryo. The *green circles* represent the position of neuromasts (NM) and the *green lines* represent the underlying nerve. The neuromasts in the head make up the anterior lateral line, whereas those along the body make up the posterior lateral line. (B) Schematic of a surface view of an individual neuromast in a 3- to 4-day-old zebrafish. The mantle cells make up the outer edge of the neuromast and the interneuromast cells are a line of cells connecting adjacent neuromasts (*dark green*). The supporting cells (*light green*) surround the sensory hair cells (*red*). (C) Cross-section of a neuromast in a 3- to 4-day-old zebrafish. The apical side of the neuromast is constricted and the hair cells (*red*) extend their hair bundles into an overlying gelatinous cupula (*yellow*). Mantle cells and interneuromast cells (*dark green*) lie at the edge of the neuromast, with supporting cells (*light green*) extending the width of the epithelium and interdigitating between the hair cells. (D) Schematic of the development of the posterior lateral line. The leading edge of the primordium (*arrow*) has migratory mesenchymal cells that crawl forward, driving the primordium along the horizontal myoseptum of the fish from head to tail. Cells in more posterior positions within the primordium begin to organize into rosettes, which are deposited as the primordium continues to migrate. In the deposited neuromast, centrally positioned cells become the Atoh1-expressing hair cell precursor (*yellow*) and organize the neuromast.

regrowth of larval salamander tails, Stone reported in the 1930s that replacement neuromasts, which contain new sensory hair cells, were produced from cells located in the most distal remaining neuromast [70,71]. As a result, the amphibian lateral line was used as a model for both sensory organ regeneration and hair cell regeneration for many years. The development of zebrafish as a genetically manipulatable model system has allowed more extensive investigation of the molecular control of these regenerative phenomena.

The mechanosensory lateral line is used to detect the flow of water and water pressure differences across the fish's or amphibian's body and has important roles in schooling behavior, predator and prey detection, rheotaxis, obstacle avoidance, and, potentially, communication between individuals [72–74]. Each neuromast is composed of a number of sensory hair cells and supporting cells and, at the outermost edge, a layer of mantle cells (Fig. 49.4B,C). The lateral line sensory hair cells are morphologically and physiologically similar to those in the inner ear, and although there are fewer markers or identifying features, the supporting cells also appear to be similar to those in the inner ear [75–77]. During development, the lateral line system is established by several different primordia that migrate from the region of the otic placode along the head and trunk, progressively depositing clusters of cells that organize into neuromasts (Fig. 49.4D). Whereas the migrating primordia establish an initial series of neuromasts, additional, new neuromasts arise from latent, multipotent interneuromast cells that are also deposited by the initial primordia as well as from budding of new neuromasts from existing ones, ultimately to form stitches, or linear arrays of neuromasts, along the body [70,71,78–80]. Once a neuromast begins to develop, the initial formation of hair cells is coordinated by *atoh1* and Notch signaling, similar to the development of hair cells in the vertebrate inner ear (see earlier discussion). Cells in the center of each new neuromast acquire *atoh1* expression, becoming hair cell precursors, and those cells use Notch and fibroblast growth factor (FGF) signaling to suppress hair cell precursor fate in neighboring cells [80–82]. The hair cell precursors then divide using planar polarity cues oriented during the migration of the primordium, such as *vangl2*, to produce pairs of hair cells oriented in the opposite direction; the hair bundles and kinocilia are oriented 180 degrees away from each other, with the kinocilia of both located closest to the center of the plane of division [83,84]. The similarity of the genes, signals, and patterns used to develop sensory hair cells within the lateral line and the vertebrate inner ear has suggested that studying the robust regeneration that occurs in the lateral line will provide valuable insights into the mechanisms that control (and limit) hair cell regeneration in all systems.

FORMATION OF NEW NEUROMASTS FROM MULTIPOTENT PROGENITORS

As mentioned previously, the first evidence that hair cells could be regenerated from supporting cells came from early experiments in salamander tail regeneration, in which it was noted that replacement neuromasts were derived from the outermost (mantle) cells on the posterior edge of the distal-most remaining neuromast after tail amputation [70,71]. Experimentation revealed that the anterior mantle cells also had the capacity to regenerate lost neuromasts if they were rotated to be proximal to the amputation site, and that these mantle cells were responsible for the budding of new neuromasts during stitch formation. These results suggested that the mantle cells retained an intrinsic latent multipotency that could be stimulated to proliferate, forming new or replacement neuromasts [70,71,78,85–87]. It has also been suggested that macrophages responding to the injury may have an important role in initiating regeneration [87], and that the regenerative primordium may reexpress markers used in the developmental primordia [88], but little is known about the signals that initiate either neuromast budding or regeneration from the mantle cells (Fig. 49.5).

In addition to the mantle cells, it has been suggested that interneuromast cells, which are deposited by the developmental primordia between primary neuromasts, may also serve as a pool of multipotent progenitors capable of forming new or replacement neuromasts. In fact, several studies suggested that the glia ensheathing the lateral line nerve, which runs beneath the lateral line, suppress the interneuromast cells from forming new neuromasts, and that interstitial growth may therefore come from de-repressed interneuromast cells that escape glial inhibition [89–92]. In addition, in response to localized destruction of an entire neuromast, interneuromast cells are capable of replacing the missing neuromast, and this regeneration is enhanced by blocking the development of lateral line nerve glia [93]. Thus, there appear to be at least two populations of latent multipotent progenitors capable of producing new sense organs in the lateral line: the mantle cells and the interneuromast cells, with both populations held in check until injury or growth requirements stimulate them (Fig. 49.5A). Notably, although full molecular characterization of mantle and interneuromast cells is incomplete [94], many common markers are expressed in both populations, which raises the possibility that these are highly similar cells, even though the mantle cells are epithelial whereas interneuromast cells appear to be more mesenchymal.

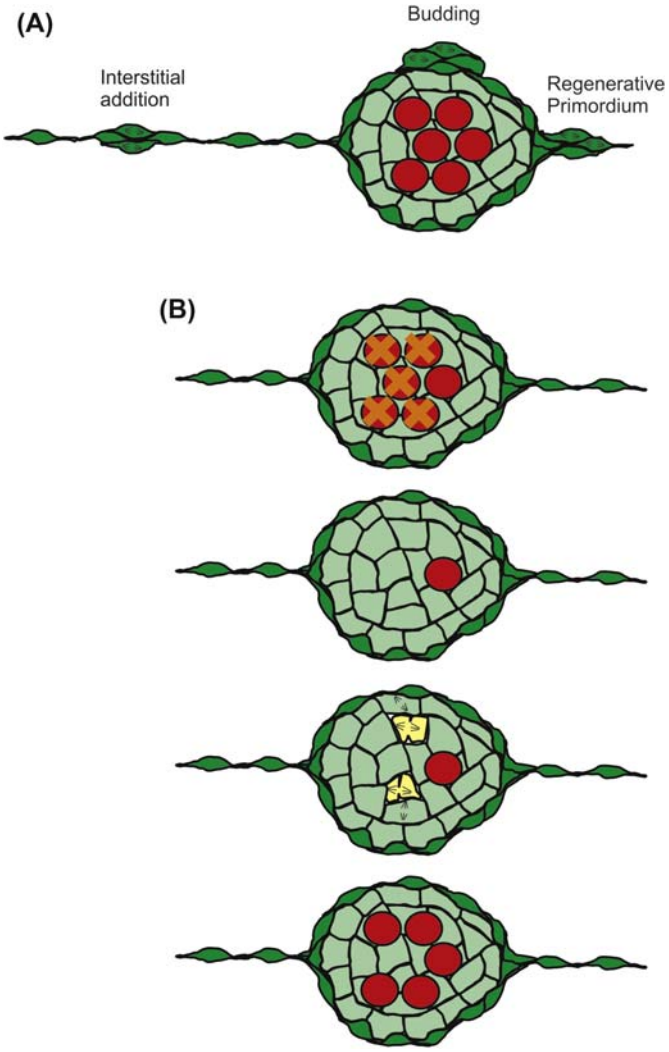

FIGURE 49.5 Models of progenitor cells in the lateral line. (A) Schematic diagram of a neuromast showing the areas where multipotent progenitors (interneuromast cells and mantle cells) can develop into new neuromasts. Interneuromast cells can divide and form a new neuromast between two existing neuromasts. Mantle cells can proliferate on the dorsoventral sides of the neuromast to bud off additional neuromasts during stitch formation. In response to amputation of the tail, the posterior neuromasts divide to become a migratory regenerative primordium that forms and deposits replacement neuromasts as it migrates into the regenerating tail. (B) Model of hair cell regeneration in the lateral line. When hair cells are ablated (Xs), they are rapidly extruded from the epithelium. Adjacent supporting cells divide symmetrically to develop into two replacement hair cells, and more peripheral supporting cells divide to give rise to replacement supporting cells.

HAIR CELL REGENERATION IN THE LATERAL LINE

The ability to regenerate entire sensory organs within the lateral line demonstrates a robust regenerative capacity; moreover, the ability also to regenerate just damaged or missing hair cells within the sensory organs makes this an important system for working out the mechanisms underlying hair cell regeneration. Like inner ear hair cells, lateral line hair cells are susceptible to damage from aminoglycoside antibiotics and chemotherapeutics such as cisplatin [95–97], and the presence of sensory structures on the surface of the fish means that hair cells can be killed simply by placing a fish in water containing ototoxins for 15–60 min. The result is rapid hair cell death and extrusion from the neuromast. The ease of applying such compounds to the lateral line hair cells, particularly in zebrafish larvae, has been leveraged to develop several screens for the discovery both of new potential ototoxins, such as copper ions and other clinically relevant compounds [98,99], as well as compounds that serve protective functions [100–103]. In addition, the surface location of the cells allows for the direct ablation of individual cells, such as by laser irradiation, and tracking of the regenerative response [104,105].

Notably, even without the addition of ototoxic compounds or other insults, there is a significant and ongoing turnover of hair cells in lateral line neuromasts [106,107], similar to that seen in avian vestibular organs [108–110]. The correlation between levels of cell death and levels of proliferative regeneration both at baseline and after the induction of damage suggests that each neuromast uses a feedback mechanism to maintain the number of hair cells within a certain range [106]. After an acute insult, loss and regeneration of hair cells occur rapidly; hair cell loss and extrusion occur within 3 h and the first regenerated hair cells appear within 12 h. Baseline numbers of hair cells are typically restored within 72 h [96,111].

Similar to results from nonaquatic vertebrates, studies in both amphibian and zebrafish lateral line have clearly identified the supporting cells as the source of regenerated hair cells [83,96,97,104–106,111,112]. Most studies suggest that regenerated hair cells are formed as a pair after the single division of a supporting cell [83,111–113]; indeed, treatment with mitotic inhibitors inhibits hair cell regeneration [97,112,114]. However, there are reports that a few regenerated hair cells may be derived via nonmitotic conversion of a supporting cell into a hair cell [105,111,115]. Because there is constant turnover within neuromasts, this nonproliferative conversion may reflect the presence of some recently produced, undifferentiated progeny at the time of lesion, or it may be tied to the specific type and level of damage induced. However, this type of regeneration is composed of only a minor percentage of new hair cells; the vast majority originate from the proliferation of nearby supporting cells. The ability of the supporting cells to regenerate hair cells extends from larval stages through to adulthood with little loss in capacity and only slight changes in the kinetics, and there is little evidence that the population of progenitors can be exhausted, because repeated rounds of neomycin still lead to robust regeneration [107,116].

Because regenerated hair cells appear to be produced as a pair from the division of one supporting cell [83], repeated rounds of regeneration would lead to a depletion of supporting cells (and loss of regenerative capacity) without proliferative replacement of the supporting cell population. However, the fates of supporting cell divisions appear to be compartmentalized based on their position within the neuromast. More central supporting cells divide to produce hair cells, whereas more peripheral supporting cells divide to renew the supporting cell population. Differences have been observed among mantle cells, with more anterior supporting cells representing a slower dividing or quiescent pool [107,117]. Mantle cells rarely appear to contribute to regeneration after limited damage, but they can contribute to the production of new supporting cells after more extensive damage such as the forced depletion of the supporting cell pool [118]. Thus, although mantle cells may be a multipotent progenitor for new sensory organ formation, they may not have regular roles in maintaining and regenerating hair cells and supporting cells, although the relationship between mantle cells and peripheral support cells, such as whether they have niche-like interactions, needs to be clarified.

PATHWAYS COORDINATING HAIR CELL REGENERATION IN THE LATERAL LINE

The ability to use both forward and reverse genetics techniques in zebrafish, the strength of various gene expression assays, as well as the ease of adding pharmacological compounds to the water has allowed extensive investigation of the pathways and genes coordinating the regeneration of hair cells after damage and comparison of those with both the developmental pathways and regeneration in other vertebrates. Transcriptomic analysis of supporting cells and mantle cells has begun to characterize genes that are active during different windows of regeneration [94,118]. Shortly after hair cell loss, Wnt, Notch, and FGF signaling pathways are each inhibited; Wnt and Notch become active in later stages of regeneration [118]. In particular, Wnt10a and the Frizzled 7b and 8a receptors are regulated during the first 5 h after hair cell ablation [118]. Consistent with these results, several studies found that the inhibition of Wnt signaling, either genetically or pharmacologically, blocks supporting cell proliferation and hair cell regeneration, whereas the activation of Wnt signaling promotes supporting cell proliferation [117,119–121]. Similarly, the inhibition of Notch signaling promotes increased numbers of supporting cells returning to the cell cycle and biases the progeny to differentiate into hair cells, whereas the activation of Notch blocks supporting cell proliferation and hair cell regeneration [111,112,117]. These pathways appear to be hierarchically arranged, because Notch inhibition activates Wnt signaling in supporting cells, likely via the loss of Notch-mediated expression of the Wnt-inhibitor *dkk2* [117,120]. This relationship is similar to that reported for Wnt/Notch control of the proliferation of supporting cells in the mouse utricle [122]. Although FGF signaling is modulated after hair cell death and is known to be important in the initial development of hair cells within neuromasts [81,82,118], the specific role of FGF signaling in hair cell regeneration is less clear. During regeneration, hair cell progenitors appear to have active FGF [123], similar to the progenitors during development, and inhibition of FGF signaling or ablation of FGF *receptor 1*-expressing support cells reduces hair cell regeneration [124]. Retinoic acid

(RA) signaling also seems to be an important inducer of supporting cell proliferation by altering $p27^{kip}$, and although RA and FGF signaling often interact, they seem to be independent during hair cell regeneration in the lateral line [123].

In addition to studies finding signals required for hair cell regeneration, negative regulators have been found. An insertional mutagenesis screen identified N-glycosylation by *mgat5a* as an important negative regulator of hair cell regeneration, because mutations in this gene had increased regeneration [125]. Although the specific pathway(s) modulated by *mgat5a* is not known, transforming growth factor-β (TGFβ) has been suggested as a candidate. Consistent with this idea, inhibition of Stat3, which is typically downstream of TGFβ signaling, enhances hair cell regeneration [126], although the early upregulation of Stat3 suggests that it may also have a role in initiating the regenerative response [118,126]. Studies have also begun to examine how epigenetic control of these signaling pathways may mediate regeneration, in particular focusing on histone methylation and its control of Wnt and FGF signaling [127,128].

Many of the most heavily studied pathways seem to mediate proliferation and/or differentiation of the hair cell progenitors, but it remains unclear what signals initiate the regenerative response, because none of the pathways that have been studied appear to be sufficient to stimulate supporting cell proliferation. Transcriptomic studies have identified other cell−cell signaling pathways including insulin, mitogen-activated protein kinase, tumor necrosis factor-α, nitric oxide, reactive oxygen species, Fat, and integrins, that are activated during regeneration, but specific analysis of the roles of these pathways during hair cell regeneration are still required [94,118]. Several of these candidate pathways, such as nitric oxide and reactive oxygen species, could be tied to pathways active in and released by dying hair cells. It has also been proposed that hair cell death may stimulate regeneration via recruitment of immune cells that secrete cytokines, because macrophages migrate to the sites of hair cell damage before the initiation of proliferation [104,105] and ablation of recruited macrophages delays hair cell regeneration [129]. Although there have been similar proposals for macrophage involvement in initiating hair cell regeneration in the avian inner ear [130−133], ablation of macrophages does not appear to limit hair cell regeneration in that system [134].

In contrast to the extensive study of hair cell regeneration in the fish and amphibian lateral line, less is known about the pathways underlying regeneration within the inner ears of fish and amphibians. Hair cells lost from the saccule, utricle, and cristae after aminoglycosides, laser ablation, or acoustic overstimulation are regenerated within 1−7 days from *sox2*-expressing supporting cells [123,126,135−137]. RA and FGF signaling appear to regulate inner ear and lateral line regeneration similarly, [123]. Interestingly, although proliferative regeneration was found in cristae and saccule [123,135], rapid recovery of hair cells in the utricle after laser ablation occurred without proliferation of the supporting cells [136], which suggests a potential capacity for direct phenotypic conversion.

OPEN QUESTIONS ABOUT LATERAL LINE REGENERATION

The lateral line system provides a unique opportunity to study hair cell regeneration at multiple levels. As described earlier, it has provided valuable insights regarding the regeneration process. One of the central questions still to be answered is whether there are unique populations of supporting cells responsible for regenerating hair cells, or whether all supporting cells have this capacity and instead environmental factors regulate which cells respond to injury. In either case, the specific gene interactions that distinguish the subset of supporting cells responsible for hair cell regeneration (in contrast to quiescent or self-renewing supporting cells) remain to be clarified. Similarly, whether there are distinctions between subpopulations of mantle or interneuromast cells in the lateral line is unclear. The ability of mantle cells and supporting cells to generate hair cells, directly in the case of supporting cells and secondarily in the case of mantle cells, suggests some common stem cell−like properties, but also important differences in their control. The separation of hair cell production and self-renewal of supporting cells into separate populations of symmetrically dividing cells is different from the mixture of symmetric and asymmetric (i.e., producing both hair cells and supporting cells) divisions reported in hair cell regeneration in the chick [18]. Whether this represents a unique adaptation of the neuromast or is a general property of hair cell regeneration in fish will require more careful examination of regenerative proliferation in the fish inner ear. Similarly, open questions still surround the potential role of nonproliferative regeneration among the lateral line, the utricle, and the saccule: in particular, whether the differences are more tied to the levels of damage that is induced, relate to different temporal effects, or reflect differences among these sensory organs.

CONCLUSIONS

All vertebrates use sensory structures containing mechanosensory hair cells to perceive sound, motion, and gravity. Although the overall structure of both the hair cells and their surrounding sensory epithelia appear to be largely comparable among orders, only mammals have lost almost all ability to regenerate hair cells. As a result, humans and all other mammals have the potential to develop permanent deficits in auditory and/or vestibular function. The biological basis for the loss of regenerative ability in mammals remains unclear.

In an effort to understand the molecular bases for hair cell formation and regeneration, two general strategies have been developed: an examination of the development of hair cells in mammals and studies of regeneration in other vertebrates, in particular zebrafish. Results from both of these studies have identified general concepts that appear to apply to hair cell formation and regeneration in all vertebrates. Hair cells typically arise from surrounding supporting cells that become activated in response to injury. The nature of that response can vary based on the extent of the injury and the cells that respond. In some cases, supporting cells directly convert into hair cells, whereas in more severe situations, different classes of supporting cells may reenter the cell cycle to generate new progenitor cells before differentiation. In both developing and regenerating epithelia, the number of cells that develop as hair cells is mediated through the Notch signaling pathway, with developing hair cells expressing ligands that bind to and activate Notch in neighboring cells preventing those cells from developing as hair cells. Similarly, the transcription factor Atoh1, which is inhibited by the Notch pathway, acts as a positive regulator of hair cell fate for all vertebrate hair cells. Based on these results, efforts have been made to manipulate both Atoh1 expression and/or the Notch pathway to induce hair cell regeneration in a mature mammal auditory organ. The results of those experiments are equivocal. Some studies suggest possible regeneration in animal models whereas others have concluded the opposite. Although subsequent work will be required to determine whether these or other factors or pathways hold the key to inducing hair cell regeneration in a mature mammalian epithelium, progress that has been achieved has been remarkable and suggests that a clinical therapy for hearing loss may be developed in the not too distant future.

CLINICAL TRIAL

Based on the results of some of these experiments, a multicenter Phase I clinical trial to examine the safety of introducing Atoh1 into the inner ears of patients was initiated in October, 2014 (NCT02132130) by Novartis. The general concept of the trial was to use an adenoviral vector to drive expression of Atoh1 in the ears of patients with profound hearing loss. As a Phase I trial, the primary end points of this study were concern regarding patient safety and the ability to tolerate the adenoviral injections. However, some patients may have been examined for possible recovery of auditory function. Although the results of this trial will not be available for some time, its approval marks the first step toward the application of gene therapy to the inner ear. As discussed, the ability of Atoh1 to induce hair cell formation in cells of an adult ear is probably limited, but if the results indicate that gene therapy can be applied safely to the inner ear, these results have the potential to usher in a series of clinical trials based on manipulations of the signaling pathways discussed in this chapter. Which of these pathways, if any, have the potential to induce a meaningful recovery of function remains to be determined. Nevertheless, that a trial of this nature has been initiated is a remarkable event in and of itself.

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CHAPTER

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Craniofacial Regenerative Medicine

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INTRODUCTION

The craniofacial region is composed of several tissue types and a number of niche environments. As such, the need to regenerate injured or diseased tissues requires strategies for engineering both hard and soft tissue, taking into account their surroundings. Tissue engineers have made great strides developing techniques that support and encourage new tissue growth. However, it is imperative that one first appreciates the unique characteristics associated with the defect. With this knowledge, health care providers can select specific approaches that optimize aspects of tissue engineering technology. Additive manufacturing (AM) techniques have become more popular in the clinical setting, and attention has turned to how patient-specific models can be used to plan and execute surgical care whereas patient specific implants can be leveraged to enhance tissue regeneration further within the craniofacial region. This chapter will discuss the craniofacial environment, review current clinical reconstructive practices, and highlight bone tissue engineering strategies with applications in craniofacial reconstruction.

UNDERSTANDING THE CRANIOFACIAL REGENERATIVE ENVIRONMENT

Several types of craniofacial defects are associated with different environments; the characteristics of each must be considered if predictable regenerative medicine results are expected. Although comprehensive descriptions of each environment are incomplete, some of the most important features have been identified through a detailed study of the normal development of tissue types and regenerative technologies based on a reproduction of embryological and remodeling biology. Using bone engineering as an example, defect characteristics exist such as [1]:

1. the resident population of pluripotent or multipotent stem cells available for differentiation;
2. vascularity of the defect and the ability of the newly formed tissue to undergo neovascularization;
3. the activity of critical genes, growth factors, and signal transduction agents that mediate tissue formation and remodeling;
4. physical features of the defect that promote tissue formation, including available space and naturally occurring scaffolds;
5. mechanical influences on the defect, including types and magnitude of loads; and
6. interactions between epithelial and mesenchymal elements.

In addition to these features, the ability of different craniofacial defects to undergo successful reconstruction is affected by the cause and the presence of infection. We will examine several common defects with different

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environmental characteristics to illustrate the need for customized regenerative strategies and why techniques for regeneration work for some defects but not for others.

One of the smallest and most challenging defects to regenerate is the periodontal apparatus that surrounds an erupted tooth and is responsible for its support. The periodontium is composed of an epithelial gingival cuff supported by mesenchymal connective tissue covering the alveolar bone, which forms the tooth socket encasing the root(s). The connective tissue fibers that attach the cementum lining of the tooth root to the socket walls are known as the periodontal ligament; within these fibers lies a network of vascular channels and neural elements with associated cells in various stages of differentiation. Loss of the periodontium typically follows chronic inflammation caused by the accumulation of bacteria- or virus-laden biofilms on the surface of a tooth and root (Fig. 50.1) [2].

The resulting infection results in osteolysis creating a pocket between the alveolar bone and overlying gingival soft tissue and in loss of the periodontal ligament attachments between the bone and root surface cementum (Fig. 50.1). This defect can have single or multiple bony walls. Defects with multiple bony walls constitute more protected environments and regenerative technologies are more successful in these circumstances. Complete restoration of the composite structures within periodontal defects remains elusive, but certain treatment strategies are required to achieve any measure of regenerative success. An appreciation of these factors provides general guidelines for the successful reconstruction of craniofacial defects that can be applied broadly.

To begin with, physical removal of the biofilm covering all surfaces of the defect is necessary. Systemic and local delivery of broad-spectrum antibiotics against periodontal pathogens are also beneficial [3,4]. The effect of infection and inflammation on bone formation is a complex topic that acknowledges the important role of proinflammatory mediators in initiating the coordinated processes responsible for bone regeneration. Some important mediators of early inflammation include interleukin (IL)-1, IL-6, tumor necrosis factor-α, and eicosanoids such as prostaglandin (PGE) (e.g., PGE_2). Evidence of the role of these mediators is provided by knockout animal studies or the observed effects of anti-PGE_2 medications such as nonsteroidal antiinflammatory drugs, which result in compromised bone formation. After these initial events, the activity of proinflammatory mediators abates and a rise in local levels of antiinflammatory mediators such as resolvins, protectins, and lipoxins (autacoids) is responsible for countering inflammation and coincides with the start of the reparative process. Both eicosanoids and autacoids are derivatives of arachidonic acid, and the mechanism for a change in synthesis from inflammatory to antiinflammatory mediators has been characterized as "class switching" and occurs by activating enzymes such as 15-lipooxygenase. If inflammation in a defect site persists as a result of chronic infection, osseous regeneration is diminished. An excellent review of the topic of inflammation and bone regeneration may be found in an article by Thomas and Puleo [5].

Once regeneration begins, the kinetics of the different reparative tissues becomes important. More specialized components of the periodontium, such as the bony walls, ligament, and bone-cementum attachments, take longer

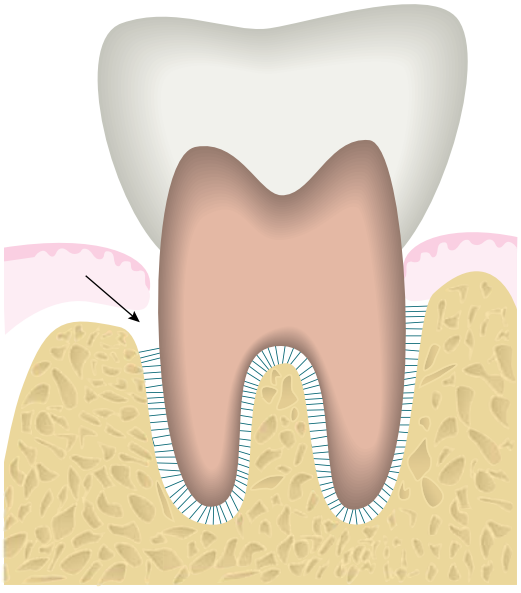

FIGURE 50.1 Periodontal pocket (marked by *arrow*) produced by loss of alveolar bone under the gingival cuff.

to form than do epithelium or connective tissue. Unless a physical void is preserved by preventing the ingrowth of epithelium and connective tissue (a technique known as *osteopromotion* by barrier techniques), periodontal regeneration is compromised. Scaffolds in combination with barrier membrane technology have been shown to be effective in restoring bone volume, and the addition of exogenous growth factors and gene therapy for the local production of growth factors are additional approaches that have been studied in preclinical studies [6].

Larger defects of the craniofacial skeleton extending beyond the periodontal defect differ from each other in a number of physical and biological ways. Defects can be intrabony and surrounded with multiple bony walls (Fig. 50.2A). In this case, the rigidity of the walls facilitates bone regeneration by protecting biological scaffolds such as blood clots, healing tissue, and neovasculature. In addition, periosteum lining the bone surfaces and the underlying endosteal surfaces are an excellent source of cells capable of differentiation under the influence of the proper growth factors. When a bony defect is segmental (Fig. 50.2B), only two bony walls remain, leading to greater instability of the skeletal structure, reduced apposition of osteogenic cell beds, and the potential for adjacent soft tissue to prolapse into the defect, later reducing bone formation. Immobilization of the bone ends (with bone plates) is important to protect early reparative activities such as the secretion of extracellular matrix and neovascularization from mechanical disruption by external loads. Filling such defects with rigid graft material (autologous, allogeneic, or alloplastic) also enhances regeneration by providing a source of living cells or tissue with inductive or osteogenic

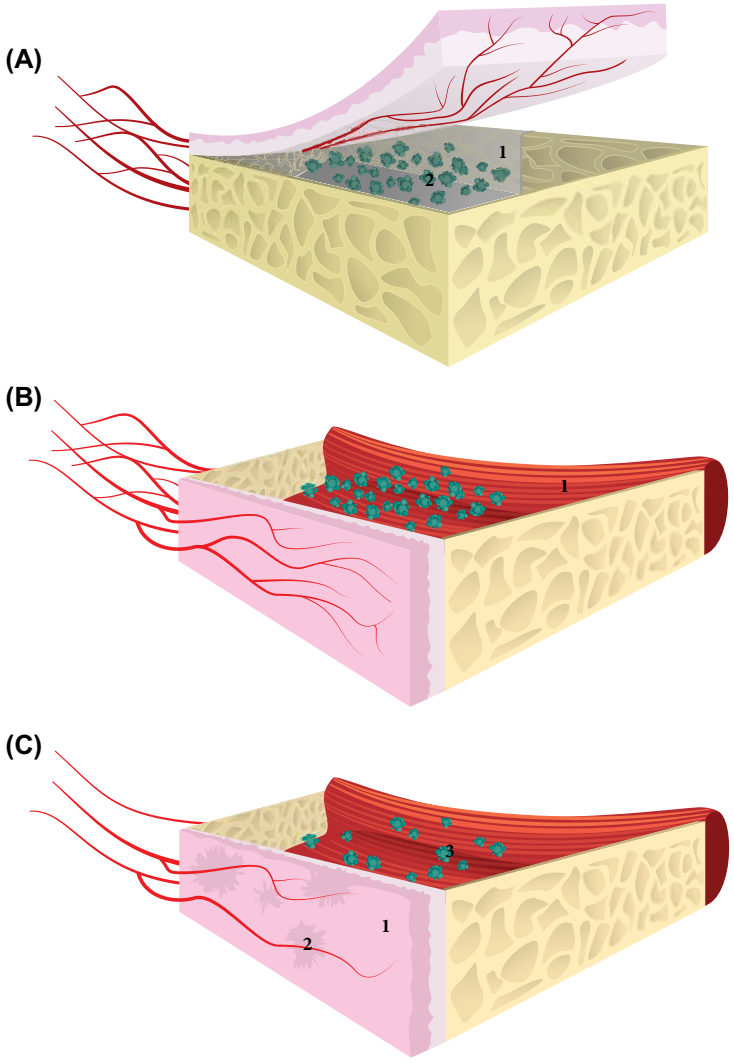

FIGURE 50.2 (A) A five-wall bone defect covered with periosteum (1) provides a protected space that contains a reparative scaffold and cells (2). (B) Segmental defects typically have a reduced number of bony walls with soft tissue (e.g., muscle [1]) adjacent to the defect. (C) Segmental defects with compromised vasculature typically have fewer vessels (1) in the soft tissue envelope, areas of fibrosis (2), and a reduced number of reparative cells (3).

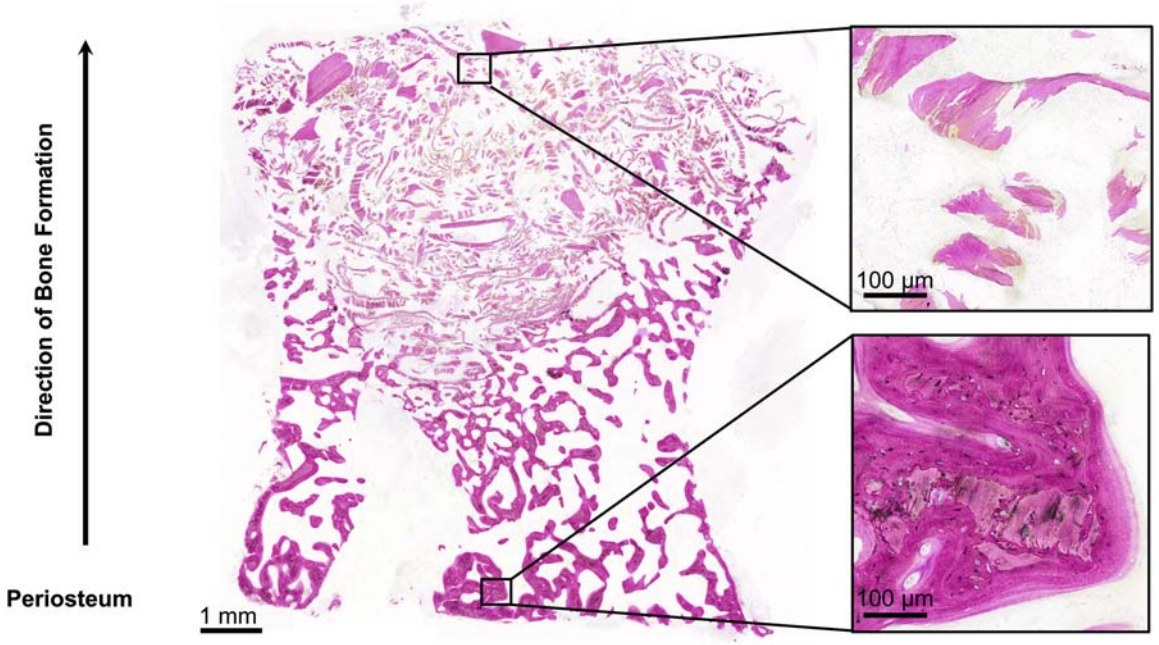

FIGURE 50.3 Histological cross-section of tissue generated within a bioreactor (initially filled with morcellized autologous graft) after implantation against the sheep periosteum for 9 weeks. High-magnification images taken adjacent periosteum and distal to the periosteum can be found on the right (magnification, 10x). *Courtesy Alexander Tatara, MS, PhD; doctoral thesis.*

capabilities. The graft can also serve as a scaffold and a barrier to the ingrowth of nonosteogenic tissue. Techniques for reconstructing segmental bone defects using tissue engineering principles are described later in this chapter.

As described in the strategies for promoting periodontal regeneration, inflammation and infection have significant roles in the regenerative capabilities of craniofacial defects. Infection of a graft site is a major factor accounting for a complication rate of 48% in a review of the literature on nonvascularized bone grafting [7]. Strategies for reducing infection through implantation of antimicrobial agents will be described in a later section.

The paradoxical relationship between inflammation and bone formation raises theoretical questions about the significance of inflammatory states, but the negative effect of prolonged inflammation on bone grafting and fracture repair is well-documented [8]. As such, it is essential to treat infected craniofacial defects by physical debridement and antimicrobial agents for healing or regeneration to occur. Newer strategies to deliver antibiotics locally using devices fabricated through tissue engineering will be described later in this chapter.

Other important conditions that can reduce the regeneration of bone include those that compromise the vascularity of the tissue surrounding a bone defect. The role of the vasculature as a source of inducible cells (pericytes), conduit for inflammatory cells (platelets, macrophages, and monocytes) and essential foundation for supporting metabolism in all living tissue is well-known [9]. When the blood supply to a defect site is compromised by therapeutic measures such as radiation therapy, comorbid conditions (e.g., diabetes), and even healing processes (e.g., scarring and fibrosis), the ability of a defect to undergo osteogenic healing is reduced (Fig. 50.2C). Efforts to reconstruct craniofacial defects afflicted by such conditions include adjuvant strategies to mitigate against the compromised vascularity such as the transfer of well-vascularized tissue beds or chemical modulators of angiogenesis. An example of the critical role of the vasculature is provided in this histological section. Fig. 50.3 illustrates bone formation within a chamber filled with autogenous bone particles placed against a vascularized periosteal membrane. New bone formation associated with neovascularization from the periosteum is seen as osteogenesis progresses from the vascularized margin upward.

CURRENT METHODS OF MAXILLOFACIAL RECONSTRUCTION

Maxillofacial reconstruction evolved from the use of nonvascularized grafting to the addition of vascularized free flaps in the late 1980s [10]. Nonvascular autogenous bone grafts were considered the reference standard for the repair of bone defects. However, successful osteogenesis from nonvascularized grafts depended on adequate soft

tissue coverage to isolate the graft site from infection, serve as a source of osteoprogenitor cells, and provide a vasculature supply. Common donor sites for bone harvest included the ilium, tibia, and calvarium [11,12]. Free tissue transfer techniques added the use of vascularized flaps anastomosed to local vessels when defect sites were contaminated or associated with compromised vascularity [13]. The impact of free flap reconstructions has been so profound that it is considered to be one of the most influential advances in head and neck surgery [14]. These flaps are harvested from areas with an axial blood supply that are isolated and anastomosed to vessels close to the defect site to produce an immediately "living" transplant. The most commonly used sites are chosen for their ease in harvest, modest donor site morbidity, and ability to include a large volume and variety of tissue types nourished from a single vascular pedicle [15]. When soft tissue is missing or when a vascularized soft tissue bed is required, the anterior lateral thigh flap or radial forearm flap can be used. For defects requiring composite soft tissue and bony reconstruction, options include the fibula, scapula, and deep circumflex iliac artery—nourished ilium flaps. Technical concerns with these techniques include the morbidity associated with graft harvest, the patency and length of vessels, the duration of surgery, and the recovery time [16]. However, the quality of reconstruction judged by the amount of bone stock is a matter of the patient's anatomy and may or may not be adequate for functional purposes.

TISSUE ENGINEERING TECHNOLOGIES CURRENTLY USED

The field of tissue engineering emerged in the early 1990s as a new way to combine the principles of biology and engineering for the development of functional tissues [17]. Since the inception of tissue engineering, several technologies have entered the marketplace offering hope for patients who have a range of conditions. In the following sections we will review advances in the field of tissue engineering that show promise for the future of craniofacial reconstruction.

Implantable Scaffolds

To allow damaged bone to be replaced with functional tissue, engineers have developed a wide range of materials that serve to stimulate the adherence and proliferation of osteogenic cells. Some of these technologies mimic the biomechanical and/or biochemical properties of native bone, whereas others try to recapitulate the anatomy [18—20].

In 1881, Sir William MacEwen of Rothesay used tibial bone wedges from three donors to reconstruct a humeral defect in a 3-year-old child, which represented the first published account of interhuman bone grafting [21]. The procedure was unsuccessful; however, later studies identified the factors affecting graft acceptance and rejection and established the parameters of allogeneic grafting. Nevertheless, despite advancements in developing allogeneic, xenogeneic, and artificial substitutes, autogenous bone grafts remain the reference standard for reconstructing segmental bone defects [22]. By definition, bone grafts can be classified as an autograft, allograft, or xenograft, depending on the source [23]. In contrast, alloplastic implants are synthetically manufactured, inorganic, and biocompatible [24]. Whereas autologous bone grafts produce the most predictable results, alloplastic materials offer several advantages over biologically derived materials. They can be fabricated to fit a patient-specific defect and the resorption rate can be controlled by adjusting material properties and compared with autologous grafts, to provide more material than can be typically harvested from a patient while avoiding a second surgical donor site. For oral and maxillofacial surgery procedures, the US Food and Drug Administration has approved a number of alloplastic materials that can be broadly divided into ceramics and polymers.

Ceramics

Numerous ceramics are available; calcium phosphate (CaP)-based ceramics represent the most widely used bioactive ceramic. CaPs offer excellent biocompatibility, possess remarkable osteoactivity, and have a chemical and crystalline structure close to those of native bone mineral [25]. Since becoming available for clinical use in 1992, CaP ceramics have been used with success within craniofacial surgery [26,27]. There are two commonly used CaP ceramics: slow-resorbing hydroxyapatite ($Ca_{10}[PO_4]_6[OH]_2$) and resorbable tricalcium phosphate (TCP) ($Ca_3[PO_4]_2$) [28]. Both of these formulations can be produced as a paste, which enables the surgeon to inject and mold the cement before final setting occurs, and which makes CaPs an attractive option for dental and orthopedic applications. Depending on the formulation of the CaP, the pore and particle size, and the metabolic activity of the

recipient site, CaP cement can take 3–36 months to be completely replaced with bone [25]. To accelerate the degradation of CaP-based scaffolds, several studies have investigated the introduction of macropores to increase the surface area of the implant and accelerate tissue integration [29–31]. The use of macroporosity has gained a lot of interest owing to improved degradation kinetics and improved tissue infiltration. Potential applications of CaP-based scaffolds have improved as several studies have investigated techniques to manufacture CaP-based scaffolds through AM techniques. This allows the engineer to improve degradation kinetics further, reproduce tissue architecture more precisely, and improve tissue infiltration [32–34]. Klammert et al. explored the ability to powder-print CaP implants to repair craniofacial defects [34]. In that study, the authors fabricated custom implants by using three-dimensional powder printing to repair defects in human cadaver skulls. The physical and mechanical characteristics of the implants were tested [34]. Whereas the four-point bending strength was in the range of 3.9–5.2 MPa, which is significantly lower than the bending strength of native compact bone (115–209 MPa), these implants could be used to reconstruct the craniofacial skeleton in non–load bearing areas [35]. A porosity of approximately 28–35% could be achieved, increasing the degradation rate and with sufficient fidelity to size and shape so that a printed implant would be able to fit the defect accurately [34].

Polymers

Polymeric-based biomaterials first emerged within the field of surgery as a material for suture and fixation devices [36]. Although numerous synthetic polymers have been tried in craniofacial surgery, only a few are extensively used in the clinical setting. Unlike natural polymers, in which degradation depends on enzymes present within the host, synthetic polymers degrade in a uniform and predictable manner by simple hydrolysis [37]. Altering the reaction environment during synthesis can precisely control the observed degradation rate, and even other properties such as mechanical properties, by altering the average molecular weight and size distribution [38]. Specifically within the field of craniofacial surgery poly(glycolic acid), poly(lactic acid), and their copolymer poly(lactic-*co*-glycolic acid) (PLGA) have been successfully used clinically [39]. Landes et al. examined the efficacy of using PLGA-based implants for maxillary and mandibular osteosynthesis [40]. In that study, five patients (6%) had an apparent foreign body whereas 75 (94%) had no observable reaction. At 24 months, histology revealed few macrophages, giant cells, and minimal bleeding residuals [40]. This demonstrated that PLGA has excellent biocompatibility but there is need for further investigation into the efficacy of bone integration. PLGA has been used as a delivery vehicle for bioactive factors. Several studies investigated the effect of using PLGA microparticles to produce a sustained release of growth factors, signaling molecules, and/or antibiotics [41–44]. These studies demonstrate the applications PLGA microparticles may have in the clinical setting.

In addition to PLGA, poly(ε-caprolactone) (PCL) has been investigated extensively within craniofacial tissue engineering owing to some of its unique properties. For example, PCL can form a wide array of biocompatible composites, blends, and copolymers [38]. If one combines PCL with other lactones, the degradation time dramatically decreases from that of the homopolymer of PCL, which is roughly 2–3 years [45]. Hollister et al. showed that the concentration of copolymer can be varied so that it has the ability to support bone formation [46]. Finally, the unsaturated linear polyester, poly(propylene fumarate), can be fabricated into scaffolds by UV-initiated photocross-linking. Although this polymer has been extensively characterized, it has been mainly used within bone tissue engineering applications [47–49].

Bioactive Molecules

Just as materials can be leveraged to promote bone growth, bioactive molecules can be introduced to craniofacial defect sites to encourage osteogenesis or angiogenesis. Growth factors are peptides that bind to receptors on a cell, leading to a cellular response. These responses vary by growth factor, cell type, the cell receptor bound, and the time course of growth factor exposure [50]. Clinically, growth factors are used in several applications. They can be employed to augment bone grafting systems, such as with the bone marrow aspirate concentrate (BMAC) technique [51,52]. Growth factors can also be used to stimulate bone formation in heterotopic sites [53–56], an important concept discussed in depth in the section on Bioreactors. Finally, growth factors can be used to form bone without using grafts, employing resorbable scaffolds such as collagen sponges, as seen with sinus augmentation procedures [57–60]. The following section focuses on bone morphogenetic proteins (BMPs) and platelet-derived growth factors (PDGFs), early animal studies, and clinical applications. Many other growth factors (i.e., transforming growth factor-β family, fibroblast growth factor family, insulin-like growth factor, and vascular endothelial growth factor) are important in tissue engineering and regenerative medicine; however, evaluation of these factors in human craniofacial defects is limited and will not be discussed here.

Bone Morphogenetic Proteins

BMPs are a large family of peptides that have a crucial role in bone and cartilage development, as well as some other functions of embryogenesis such as heart and kidney formation [61]. The family acts via two distinct type II and type I serine/threonine kinase receptors; both types are required for signal transduction. BMPs act by stimulating osteoblasts. BMP-2, BMP-4, and BMP-7 show efficacy with bone formation in vivo including in critical-sized defects [62]. The US Food and Drug Administration approved recombinant human BMP-2 and BMP-7 in 2002 for spinal fusion surgeries. In addition, BMP-14 is being tested clinically as a growth factor alternative [63]. There is controversy regarding the use of BMPs in spinal fusion surgeries; a metaanalysis showed efficacy similar to autograft but with more complications, especially when used in the cervical spine [63].

Despite controversy about the use of BMPs in spinal fusion [63], they show great potential for use in the craniofacial area [64–68]. In a rodent study, BMPs have been shown to aid in the healing of critical-sized cranial defects [64]. After loading BMP-2 into PLGA microparticles within a scaffold in a critical-sized, 1-cm defect cranial defect, total union was found in three of eight animals, and the bone volume generated significantly increased over the blank group [65,66]. Larger animals show similar trends. In baboons, a 2.5-cm, critical-sized cranial defect was formed [65]. A collagenous bone scaffold loaded with BMP-7 was introduced to the defect. Groups receiving high doses of BMP-7 (2.5 mg/g scaffold) had extensive osteogenesis, whereas those with low doses (0.1-mg/g scaffold) showed significant increases in bone growth over control animals. A box-type dehiscence defect in the edentulous region of dogs was employed to compare the use of synthetic bone substitute (SBS) and BMP-2–loaded hydrogel [66]. After 8 weeks, the animals were killed and the defects examined. Significantly increased bone volume was seen in the groups containing BMP-2 (14.7 mm^3 for the BMP-2–loaded hydrogel and only 2.01 mm^3 for the SBS-loaded hydrogel).

Several clinical studies were conducted to evaluate the efficacy of recombinant human BMPs (rhBMPs) in human craniofacial defects [67]. In 47 identified case studies in the systemic review, rhBMP-2 was shown to be effective in enhancing bone formation in socket healing and in sinus lift procedures, and BMP-14 was shown to be effective in sinus lift procedures [67]. In one maxillary floor sinus augmentation study, 160 human subjects were enrolled and treated with rhBMP-2 on a collagen sponge or autograft [57]. At 6 months, there was no difference in the change of height between the two groups (7.83 ± 3.52 and 9.46 ± 4.11 mm for the rhBMP-2 and autograft groups respectively), but the density of the bone in the rhBMP-2–treated group was significantly higher. Another clinical study analyzed the effects of rhBMP-2 on the repair of buccal wall defects [68]. Groups received rhBMP-2 (1.5 or 0.75 mg/mL) on an absorbable collagen sponge, just the collagen sponge, or no treatment. The assessment of the alveolar bone showed significantly increased adequacy (as calculated by height and width measured computed tomography [CT] scans and number of repeat procedures needed) over the control or untreated groups.

Platelet-Derived Growth Factor

PDGF is a dimer composed of PDGF A, B, C, or D joined by sulfide bonds [69]. Several homodimers (PDGF-AA, PDGF-BB, PDGF-CC, and PDGF-DD) and one heterodimer (PDGF-AB) exist. Two isotypes of the receptor exist, α and β, with different binding affinities. PDGF-BB can bind to all receptor isotypes and is often considered the universal PDGF [69]. In vitro studies have shown PDGF is produced by osteoblasts and inhibits osteoclastogenesis [70]. In vivo, PDGF is important in new bone formation and in fracture healing [70].

In humans, PDGF is expressed naturally in the course of fracture healing [71]. PDGF-A chains were found expressed by a variety of cell types throughout the healing process. PDGF-B chains were expressed in a more selective pattern: by osteoblasts at the time of bone formation [71]. Several clinical studies investigated the use of PDGF on the healing of craniofacial defects [72,73]. One study investigated intrabony periodontal defects of greater than 4 mm depth treated with β-TCP alone or β-TCP with recombinant human (rhPDGF)-BB [72]. In the group treated with β-TCP and 0.3 mg rhPDGF-BB, the clinical level of attachment was found to be greater and gingival recession was found to be less at 3 months than that of control patients who received only β-TCP. The clinical level of attachment was not significant at 6 months. Given the significant difference at the early 3-month evaluation with loss of significant differences at the 6-month appointment, rhPDGF-A-BB increased the rate of attachment in the treated groups, providing an advantage for treated patients soon after the surgery. A similar defect treated with rhPDGF-BB was analyzed for the rate of bone turnover [73]. Pyridinoline cross-linked carboxyterminal telopeptide of type I collagen, a well-known biomarker of bone turnover, was shown to be increased at the early study time points of patients treated with rhPDGF-BB, which again indicated advantages for patients with decreased recovery times. rhPDGF has also been evaluated in humans for the treatment of sinus augmentation and ridge preservation [59,60]. Both studies involved using inorganic bovine bone material with rhPDGF-BB as the treatment method, and both studies exhibited good bone growth.

Bone Marrow Aspirate Concentrate Technique

As described in the preceding sections, the principal efforts in tissue engineering have focused on stimulating cell multiplication and differentiation and the development of artificial scaffolds with tunable properties to enhance cell adhesion, vascular ingrowth, and scaffold degradation in coordination with new tissue formation. To meet the goal of reconstructing bony defects, another approach was devised to combine several of the more important contributions in tissue engineering methodology in vivo to take advantage of the body's established vascular system.

In keeping with the classical tissue engineering paradigm, the three essential components are provided with clinically available materials. This technique uses allogeneic bone to maintain space and support the ingrowth of vessels and adhesion of bone-forming cells. Differentiation and regenerative signals are provided by inductive bone proteins such as rhBMP-2, which promotes the differentiation of stem cells and migrating osteogenic cells [74]. Finally, a concentrated regenerative cell population composed of autogenous BMAC is introduced into the defect site. The aspirate contains a highly concentrated population of mesenchymal stem cells (MSCs) (Figs. 50.4 and 50.5) and migrating osteogenic cells that serve as a source of cells for differentiation into osteoblasts. By combining these agents, bone regeneration is promoted, which can lead to successful reconstruction of a bony defect (Fig. 50.6). Several studies reported the efficacy, safety, and ability of rhBMP-2 when combined with osteoconductive grafts to accomplish mandibular reconstruction [51,52]. The use of osteoprogenitor or stem cells from a bone marrow aspirate has improved results and offered another technique to reconstruct craniofacial bone defects.

Bone marrow aspirate serves as a rich and readily available source of bone-forming cells (MSCs), which otherwise would not be present in sufficient quantities in a traditional bone harvest. It is easily harvested with simple aspiration through large-bore needles and concentrated with centrifuge devices without significant donor site morbidity [75,76]. Bone marrow transplant/aspirate was first used to treat hemopoietic and oncologic diseases, but it has found additional uses as a cell source in the regeneration of other tissues in the body. Several studies have shown bone marrow−derived stem and progenitor cells to be capable of regenerating bone in both animal and human models [77,78]. BMAC has also been used with spine, long bone, and myocardial regeneration [77,79−81]. Of the mixed population of cells collected in an aspirate, Marx and Harrell suggested that CD34+, CD44+, CD90+, and CD105+ cells are the main types of osteoprogenitor cells collected in the concentrate [82].

In a comparison of osteogenic activity, Gimbel et al. studied patients undergoing alveolar cleft repair with different graft materials. A total of 69 patients were divided into three groups. Group 1 underwent grafting with bone marrow aspirate seeded onto a resorbable collagen matrix (n = 21); group 2 received autogenous cortical and cancellous bone harvested from the ilium through a traditional open approach (n = 25); and group 3 received autogenous cancellous bone that was collected from the ilium with a cannula (n = 23) [83]. Whereas alveolar bone formation was comparable in all three groups, the BMAC plus scaffold group experienced significantly less morbidity, operative time, duration of hospital stay, and cost.

A study conducted by Hendrich et al. described 101 patients treated with BMAC injections for various bone healing disturbances of the femur including necrosis of the head of the femur (n = 37), avascular necrosis (n = 32), nonunion of fractures (n = 12), and other problems (n = 20) [81]. After an average of 14 months (2−24 months),

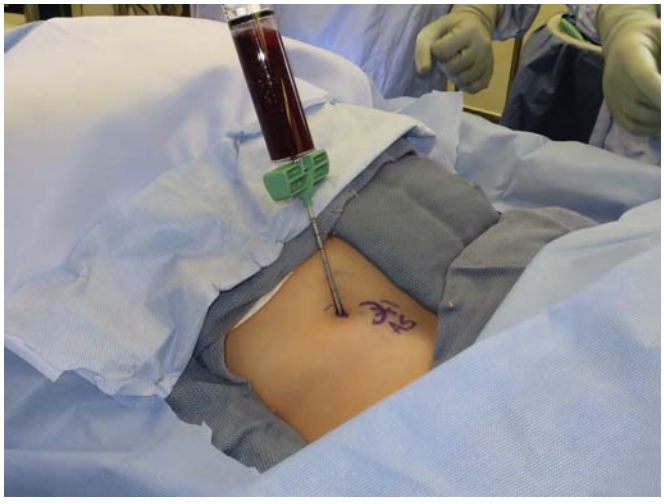

FIGURE 50.4 Aspiration of bone marrow from ilium.

FIGURE 50.5 Aspirate after centrifuging, demonstrating separation of cells from plasma and red blood cells.

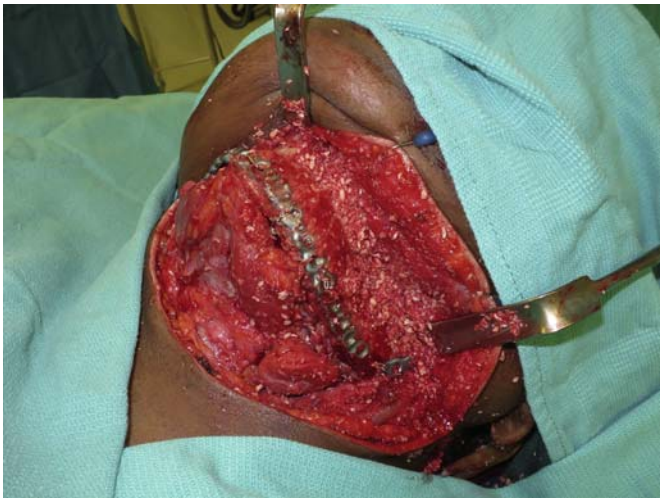

FIGURE 50.6 Tissue engineered graft for mandibular reconstruction: mixture of bone marrow aspirate concentrate, allogeneic bone, and recombinant human bone morphogenetic protein-2.

the patients were reexamined clinically and radiologically and interviewed. Of the 101 patients, only 2 required additional surgery to correct a nonunion or inadequate bone formation; no additional complications such as infections, excessive bone formation, or harvest site morbidity were observed. The researchers' conclusion was that BMAC therapy was a suitable alternative to open treatment but required additional studies to determine the full benefits of this treatment modality.

One of the most exciting applications of the BMAC technique in maxillofacial reconstruction lies in treating large segmental defects of the mandible. Marx and Harrell reported on a series of 40 patients who underwent mandibular reconstruction using this method [75]. All 40 patients achieved successful mandibular continuity that was functionally useful. Melville et al. conducted another study with five patients who underwent immediate intraoral reconstruction with the BMAC technique after resection of benign mandibular tumors [84] (Figs. 50.7 and 50.8). All patients achieved excellent bone quality both clinically and radiographically and were successful candidates for endosseous dental implant placement (Figs. 50.9 and 50.10). This case series demonstrated that composite allogeneic

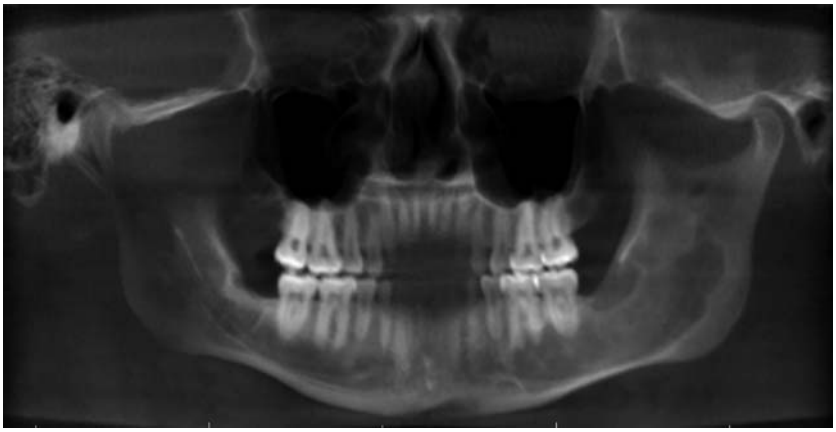

FIGURE 50.7 Orthopantomogram radiograph of benign tumor (ameloblastoma) of the left mandible.

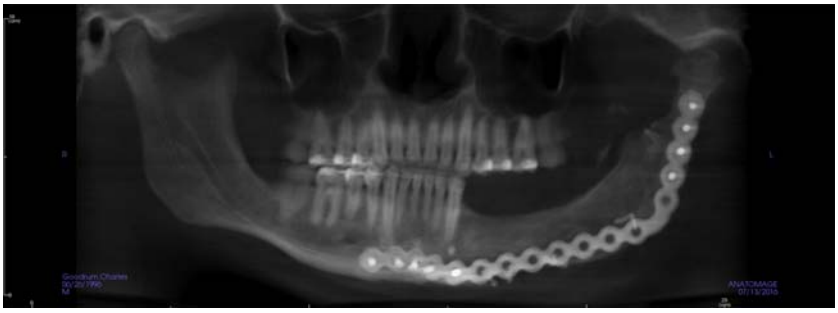

FIGURE 50.8 Orthopantomogram radiograph 1 year after resection and reconstruction with bone marrow aspirate concentrate, allogeneic bone, and recombinant human bone morphogenetic protein-2.

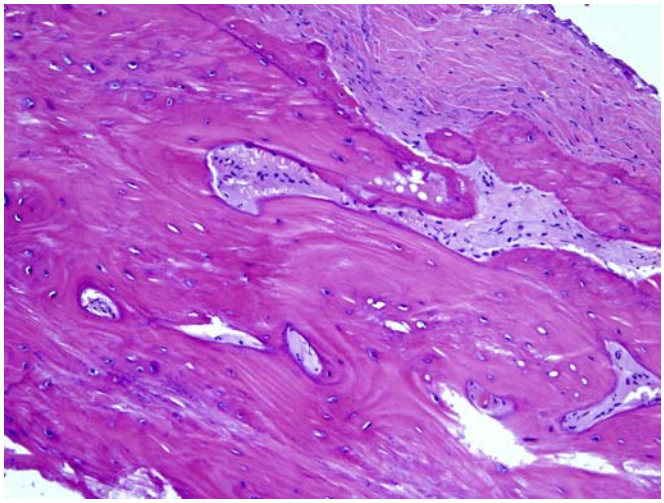

FIGURE 50.9 Histology of tissue engineered mandibular reconstruction bone (hematoxylin-eosin stain., 10× magnification). Normal reactive bone with regular trabecular pattern with fibrosis. No remnants of cadaver bone were seen after 8 months.

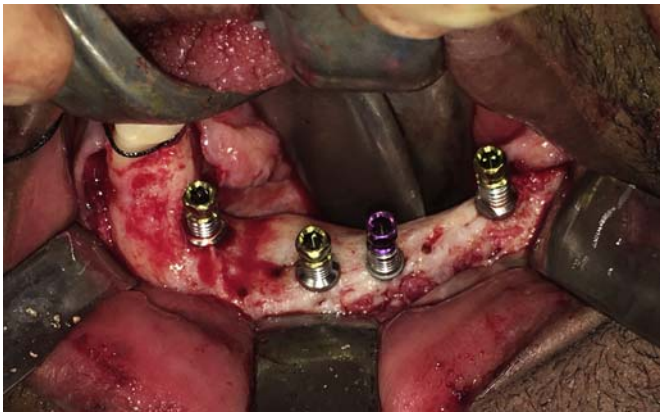

FIGURE 50.10 Placement of dental implants in tissue engineered reconstructed bone. Alveolar ridge demonstrating excellent height and width for placement of four 5 × 13-mm implants.

bone, BMAC, and rhBMP grafts constitute an effective and predictable technique for immediate reconstruction of mandibular continuity defects. In addition, there was no donor site morbidity, intraoperative time was reduced, hospital stay was shorter, and total costs were lower compared with more traditional methods of mandibular reconstruction.

Bioreactors

If the desire is to use living biological tissue and avoid donor site morbidity, another strategy employs bioreactors to grow tissues in geometries matching the defect in either in vitro or in vivo environments [85]. In vitro bioreactors have been used to engineer tissues such as the temporomandibular joint (TMJ) or ear perichondrium [86,87]. Using human MSCs obtained from fresh bone marrow aspirate, researchers were able to observe confluent layers of lamellar bone and osteoids on the decellularized bone scaffold [86]. This required the use of a bioreactor of the same size and dimensions as the TMJ and a constant flow of media across the scaffold. Other types of in vitro bioreactors are also capable of generating viable tissue. A rotating wall bioreactor has been successfully used to cultivate elastic cartilage [87]. Tissue obtained from patients were differentiated into cartilage progenitor cells and injected into a porous scaffold of collagen, hydroxyapatite, and chondroitin sulfate. After culture for 6 weeks, differentiated chondrocytes and elastic fibers were observed in histology.

Although the formation of tissue engineered grafts is possible in vitro, it is important to consider how these tissues will obtain nutrients in vivo. For small tissues, the transport of nutrients and byproducts may be adequate; however, for large tissues, the diffusion rates may not be adequate for the tissue to survive [88]. By introducing vasculature into the construct, cells deep within the tissue can receive appropriate nutrition and gas exchange. In vivo bioreactors have been used to generate vascularized flaps that match the geometry of the defect [85,89–91].

Using strategies previously discussed, in vivo bioreactors can be designed to encourage bone growth in a chamber of desired dimensions. As with osteopromotion strategies, growth factor incorporation or cell seeding can be used directly within the defect to encourage bone growth. These techniques can be used to grow bone in bioreactors in animal models [89–91]. Using MASTERGRAFT, a clinically available scaffold consisting of hydroxyapatite and β-TCP, bone can be grown adjacent to rib periosteum in a large-animal model [89]. Because of the close proximity to the intercostal arteries and veins running below the ribs, tissue from the bioreactor chambers could be removed with the nearby vasculature and transferred to a mandibular defect as a vascularized flap. Whereas some chambers were filled with autograft (requiring harvest from elsewhere in the body), those filled with synthetic graft allowed for the formation of mineralized tissue and reconstruction of large mandibular defects. Incorporation of growth factors to in vivo bioreactors can also be used to promote advantageous ectopic bone growth [90]. BMP-7 was added to Bio-Oss (a scaffold derived from bovine bone) and placed in a pouch created within the latissimus dorsi muscle. After 6, 12, and 24 weeks, tissue was harvested and analyzed. Analysis showed the formation of new bone with vascular supply that could be used for mandibular reconstruction. As an alternative to loading scaffolds with growth factors, cells can be seeded onto scaffolds to promote tissue growth in in vivo bioreactors [91]. Porous, degradable PCL scaffolds were seeded with MSCs from neonatal rats. After 4 weeks of in vitro culture, the cell-seeded scaffolds were implanted in the omenta of rats. Four weeks later, the scaffolds were harvested

and mineralization was observed throughout the scaffold. However, the formation of vasculature allowing for flap transfer was not investigated.

With strong support for the efficacy of in vivo bioreactors in animal models, several bioreactor systems have been used clinically [53–56,92]. The earliest reported case involved angle-to-angle mandibular resection owing to a recurring ameloblastoma [53]. The mandibular-shaped chamber was loaded with autologous bone harvested from the iliac crest and BMP before implantation within the fascia above the scapula. After 4 months, the flap was harvested and transferred with vasculature to the mandible. Competence was restored for liquids and solids, but the patient did not regain the ability to swallow solids. Another clinical case study involved Bio-Oss, bone marrow, and rhBMP-7 within a titanium mesh [54]. The titanium mesh was shaped using CT scans to ensure a close match with the native patient architecture. After 7 weeks within the latissimus dorsi muscle, the mineralized tissue, titanium mesh, and adjacent vasculature were transferred to the mandible. The flap showed remodeling after transfer and the patient had an improved level of mastication after reconstruction completed. Unfortunately, after 13 months, the mesh fractured and the gingiva became disrupted, resulting in infection and necrosis of the mandible replacement [92]. The patient died soon thereafter of cardiac arrest. Many of the human in vivo bioreactor studies require the need for morcellized autograft or bone marrow. Hydroxyapatite with rhBMP-7 was capable growing mineralized tissue in the pectoralis muscle [55]. This tissue was then transferred to the mandible. The patient could talk and eat food; however, 5 months after transfer, the flap became infected. Another clinical study used CT images to shape a titanium mesh cage [56]. Bone blocks, rhBMP-2, and bone marrow aspirate were placed within the cage before implantation in the gastric omentum. After 3 months within the gastric omentum, the free tissue flap was harvested and used to reconstruct the mandible. Three months after transfer, the patient could talk and eat normally.

Adjuvant Therapies

Antibiotics

When repairing craniomaxillofacial tissue defects, proximity to bacterial flora is an important consideration. The oral cavity hosts over 500 bacterial species, including many species of *Streptococcus* [93], and the nasal cavity has been shown to be colonized by *Staphylococcus aureus* in 21% of patients at admission [94]. Although native bacteria generally do not cause problems, trauma or the following reparative surgery can shift the bacterial balance in favor of pathogenic bacteria or permit the passage of bacteria to previously aseptic sites. In facial fracture repair, infection rates can be as high as 42% if no antibiotics are received before surgery [95].

The use of cefazolin sodium has been shown to reduce the rate of infection to 9% during the surgical repair of facial fractures [95]. Although antibiotics are commonly provided systemically before many craniofacial surgeries, a systemic review and metaanalysis of endoscopic sinus surgeries showed no statistical difference in infection rate between patients who receive antibiotics and those who do not [96]. The authors noted that the amount of available data may have limited the statistical power of their analysis, however. For clean-contaminated wounds such as those commonly seen in patients with head and neck cancers, antibiotic prophylaxis becomes mandatory [97]. In cases in which the upper aerodigestive track is entered, the infection rate is much greater. In addition, these infections are often polymicrobial, and the chosen antibiotic should cover aerobic, anaerobic, and gram-negative flora [97].

From a tissue engineering perspective, local antibiotic release is an exciting avenue for exploration. A scaffold designed to promote bone growth, by material properties or the incorporation of bioactive molecules, could potentially deliver antibiotics to a local area, thereby alleviating some of the systemic effects of the antibiotic. Using an infected composite defect in a rabbit mandible, clindamycin-loaded poly(methyl methacrylate) space maintainers were shown to clear infection [98]. The study investigated both burst and extended release kinetics, but the inoculated bacteria were not recovered from any group. Several clinical products exist for local antibiotic release [99]. These clinically available products include gels, chips, fibers, and polymers consisting of a range of antimicrobials such as tetracycline, metronidazole, and doxycycline. After several days of controlled drug release to the site of the periodontal disease, degradation occurs for many of these products whereas others must be surgically removed [99].

Although the species distribution of craniofacial infection has not changed much over the years, the antibiotic resistance of these organisms is growing [100]. The production of β-lactamases has limited the efficacy of penicillin against some gram-negative species, and others are increasingly resistant to clindamycin. This growing resistance has led researchers to explore other potential methods of combatting infection [101,102]. Human saliva contains antimicrobial peptides that can cause bacterial cell death [102]. Although over 45 such peptides have been found,

efficacy in human trials has been limited. Antimicrobial peptides can be designed with different functional groups and can be modified to bind hydroxyapatite selectively [101]. A modest minimum inhibitory concentration (125 μg/mL) was seen against *Streptococcus mutans* and *Lactobacillus acidophilus* when bound to hydroxyapatite. Although this particular antimicrobial peptide was designed to bind to tooth surfaces to combat biofilm formation, the in vitro studies were conducted on hydroxyapatite scaffolds, a major component of the CaP cements discussed earlier.

Patient-Specific Technology

Tissue engineering scaffolds have classically been fabricated from techniques such as electrospinning, phase separation, gas foaming, and particulate leaching [103]. Although researchers have worked to optimize and refine these techniques further, there are intrinsic limitations to the architecture and topography that can be achieved with these conventional techniques. Furthermore, techniques such as electrospinning require the use of organic carcinogenic solvents [104]. The field of tissue engineering has embraced AM or rapid prototyping techniques to bypass these limitations and produce scaffolds with superior reproducibility, more sophisticated structural features, and even patient-specific constructs [105]. Tissue engineers have leveraged AM to control the internal architecture of scaffolds, whereas surgeons have adopted AM to produce solid biomodels that reproduce the patient's anatomy. The field of craniomaxillofacial surgery first adopted AM techniques to produce patient-specific models for surgical planning [106]. This proved to be an invaluable tool for surgeons, because an operation could be planned beforehand. In addition, it allowed the surgeon to predict physical outcomes of the procedure [106]. AM techniques have been extended to produce patient-specific implants. These custom implants precisely fit within a defect site, reducing surgical time and improving aesthetics [107,108].

To generate a patient-specific implant, a fine-cut CT study must be obtained that is acquired according to a special protocol. The radiographic data are first processed with software such as 3D Doctor, to creates a three-dimensional model of the defect. This model is then transferred to design software that allows the engineer to create the implant design. The implant is manufactured from this dataset by subtractive or AM techniques before sterilizing, packaging, and delivery to the surgeon. Initially, the fabrication of patient-specific implants was hindered by limitations within the fields of design and manufacturing. However, technological advances have led to customized cranial, dental, and facial implants in addition to space holders for grafts [58,109–111].

Chacon-Moya et al. published a case report of a 63-year-old female patient diagnosed with esthesioneuroblastoma [111]. The patient underwent an anterior craniofacial resection and radiotherapy. After treatment, the patient developed secondary complications of osteomyelitis and osteoradionecrosis that resulted in loss of the anterior cranial vault [111]. After resolution of the infection, the patient underwent frontal bone reconstruction using a computer-generated poly(ether ether ketone) (PEEK) implant. The patient was observed for 5 months after the PEEK implantation and no complications were observed. This case serves to demonstrate that reconstruction using PEEK implants is an excellent option in patients with large bone defects. Alternatively, computer-aided design–based technology can be leveraged to fabricate customized jigs and cutting guides (Fig. 50.11A and B). This allows the surgeon to create osteotomies in the fibula, resulting in an autogenous graft that approximates the defect contour (Fig. 50.12). Hou et al. used three-dimensional model simulation to contour vascularized fibular osteomyocutaneous flaps to repair mandibular defects in 15 patients [112] (Fig. 50.13). In this report, all patients experienced uneventful healing and were satisfied with the functionality and esthetics when questioned 6 months after surgery. These examples further illustrate the beneficial role AM has within the field of craniofacial surgery.

CONCLUSION

The field of tissue engineering continues to make important strides toward the goal of producing biological tissue of high functional and esthetic fidelity to replace anatomical structures lost to trauma or disease. The field has developed sophisticated biomaterials that leverage manufacturing techniques, characterized and manufactured cell signaling proteins, and developed strategies to deliver therapeutic doses of antibiotics locally to improve tissue regeneration further. In addition, clinicians trying to apply this technology have gained new appreciation for the underlying cause of a defect, which has a significant role in the success of tissue regeneration. Although there have been significant advancements in the field, new directions of investigation into regenerating composite, vascularized, and innervated constructs are required to account for all the tissue types present within the craniofacial region.

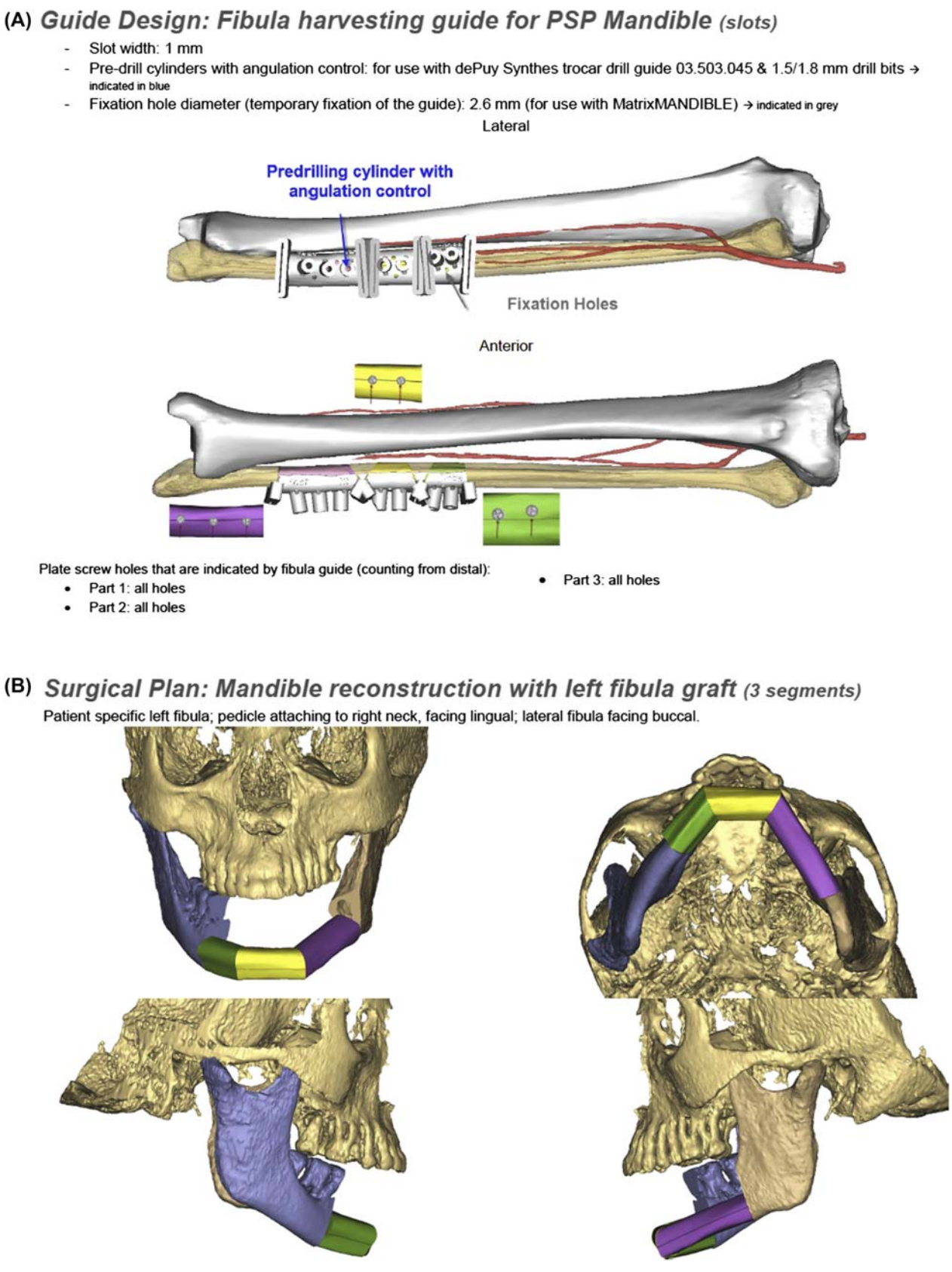

FIGURE 50.11 (A) Virtual surgical planning (VSP) matches the geometry of the fibula to the planned mandibular defect for reconstruction. (B) VSP plan shows final mandibular reconstruction using the fibula, which has been contoured to fit the defect through osteotomies and ostectomies.

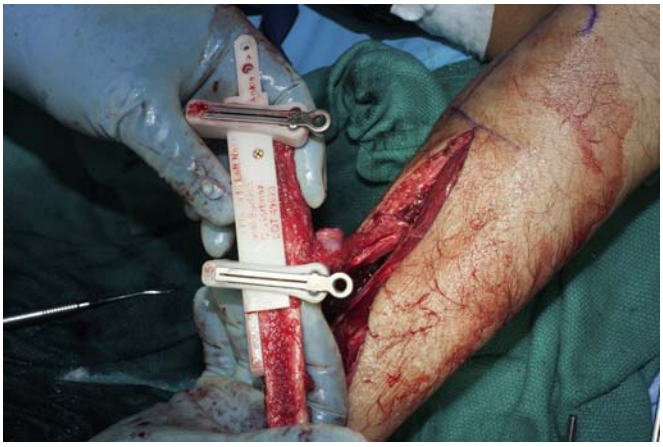

FIGURE 50.12 Custom-fabricated plastic jigs applied to the fibula to guide the creation of osteotomies to contour the bone.

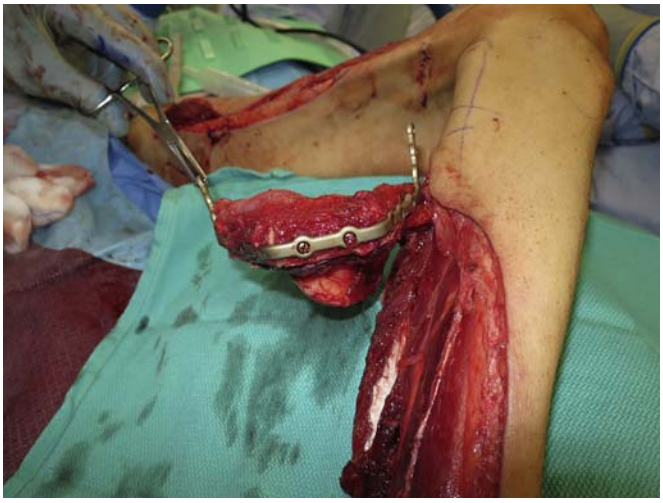

FIGURE 50.13 Fibula contoured with custom jigs is plated to maintain its shape before transplantation to the mandibular defect.

List of Abbreviations

AM Additive manufacturing
BMAC Bone marrow aspirate concentrate
BMPs Bone morphogenetic proteins
CaP Calcium phosphate
CT Computed tomography
MSC Mesenchymal stem cells
PCL Poly(ε-caprolactone)
PDGF Platelet-derived growth factor
PLGA Poly(lactic-*co*-glycolic acid)
rhBMP-2 Recombinant human bone morphogenetic protein-2
rhPDGF Recombinant human platelet-derived growth factor
TCP Tricalcium phosphate
TMJ Temporomandibular joint

Acknowledgments

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CHAPTER

51

Dental Tissue Engineering

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INTRODUCTION

Dental caries, trauma, genetic disorders, and periodontal diseases may cause damage and loss of dental tissues, and ultimately loss of the whole tooth [1]. Despite advancements in dental therapies, loss of dental tissues and teeth is a concern both to individuals and to their professional health care providers and remains a highly prominent public health issue [2,3]. It was reported that the public health burden associated with dental treatment of root canals represents a significant fraction of all expenses related to dental care, which totaled $100 billion in 2009 in the US alone [3]. Approximately 150 million adults experience tooth loss, and it is expected that over 10 million new cases of edentulism will have arisen in one decade [2]. Another highly significant population and of particular concern to the field of regenerative medicine and the US Department of Defense is the military. In 2000, a cost of $1.9 billion was estimated for active duty personnel and $203 million for recruits; periodontal disease accounted for the greatest proportion of active duty treatment costs (47%) and oral surgery for the greatest proportion of recruit treatment costs (32%) [4]. Therefore, the health of both military and civilian populations would significantly benefit from the development of new, improved, and alternative functional dental tissue replacement therapies.

Treatments for deep carious lesions include pulp capping or partial pulp amputation to preserve the pulp tissue [5,6]. However, in the case of irreversible pulpitis, root canal treatment or extraction of the tooth is necessary. Root canal therapy involves complete removal of the infected pulp tissue and replacement with inert material [7]. There are some limitations associated with root canal treatment, including pulp tissue devitalization and excessive enlargement of the pulp chamber; in addition, debridement of infected root canals and the preparation of a post to support a synthetic tooth crown can increase the possibility of tooth root fracture and tooth loss. Dental prosthetic procedures such as dental implant placement have been used as tooth replacement therapies for many centuries [8]. Dental implants function through osseointegration, which is direct integration of the implant with the surrounding alveolar bone [9]. Dental implants have improved significantly over the years, based on innovations from basic and translational research, material sciences, and clinical techniques [8]. However, they are not equivalent to natural teeth in either function or aesthetics. They lack periodontal and cementum tissues, which function to cushion and modulate the mechanical stresses of mastication [9]. These disadvantages have driven an ongoing search for alternative strategies to overcome both the need for root canal and dental implant treatments. This search has led to the development of dental tissue engineering (TE) technologies, which consist of combined TE and clinical approaches to bioengineer dental tissues and whole teeth. Dental TE has demonstrated the potential use of dental stem cells (DSCs), biodegradable scaffolds, and bioactive agents such as growth factors (GFs) to control the spatial and temporal organization of regenerated dental tissues [8,10]. It is expected that dental TE will soon emerge as the preferred solution to root canal and dental implants therapies. Here, we summarize advances in dental TE to regenerate dental tissues including dental pulp, dentin, periodontal tissues, alveolar bone, and whole teeth.

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TOOTH DEVELOPMENT

Tooth development is a tightly regulated process mediated by dental epithelial–mesenchymal cell interactions, Fig. 51.1. The dental mesenchyme and dental epithelium are derived from the neural crest and the ectoderm, respectively [11]. Tooth organogenesis is regulated by a complex and intricate network of cell–cell signaling, gene expression, and GF signaling pathways [12]. Progress in elucidating the roles of molecular signals in natural tooth development has shed light on new approaches to control better the cell migration, growth, and differentiation of regenerated tissues [8,12,13]. It is known that at least 12 transcription factors are expressed in odontogenic mesenchyme [12], and more than 200 genes have been identified that are expressed in the oral epithelium, dental epithelium, and dental mesenchyme during the initiation of tooth development [14]. Tooth organogenesis is initiated by the formation of the dental lamina within the dental epithelium. The dental epithelial placode exhibits localized proliferative activity leading to dental epithelial outgrowths into the ectomesenchyme. The developing tooth organ proceeds through bud, cap, and bell stages [11,12]. In the bell stage, species-specific cusp patterns emerge, forming either a single or a multicusped tooth. The bell stage is followed by the differentiation stage, in which final growth and matrix secretion occur as the inner enamel epithelium differentiates into enamel-producing ameloblasts, whereas adjacent dental mesenchymal cells differentiate into dentin-producing odontoblasts [11,13].

The dental pulp is the soft connective tissue in the center of the tooth, which is enclosed by dentin [12]. Dental pulp has a variety of functions: (1) to support nerves that provide sensitivity to the tooth, (2) to nourish the avascular dentin, and (3) to produce the dentin that surrounds it. Dental pulp differs from other craniofacial structures in that it is extensively vascularized and innervated. Pulp vascularization is established by vasculogenesis during embryonic development of the tooth, and angiogenesis occurs during regeneration and therapeutic processes. Pulp innervation occurs at a relatively late postnatal stage and is innervated primarily by nociceptors [15]. Dentin is a resilient and elastic tissue that forms the bulk of the tooth, supports the enamel, and compensates for the brittleness of the highly mineralized enamel tissue. Dentin is a sensitive tissue that is capable of limited self-repair [12]. Odontoblasts and dental mesenchymal cells present in the tooth pulp can be stimulated to deposit new dentin in response to mechanical injury, termed reparative dentin. Enamel is the most highly mineralized tissue in the body; it consists of greater than 96% hydroxyapatite (HA) and exhibits a complex crystalline lattice organization [11,12]. Ameloblasts,

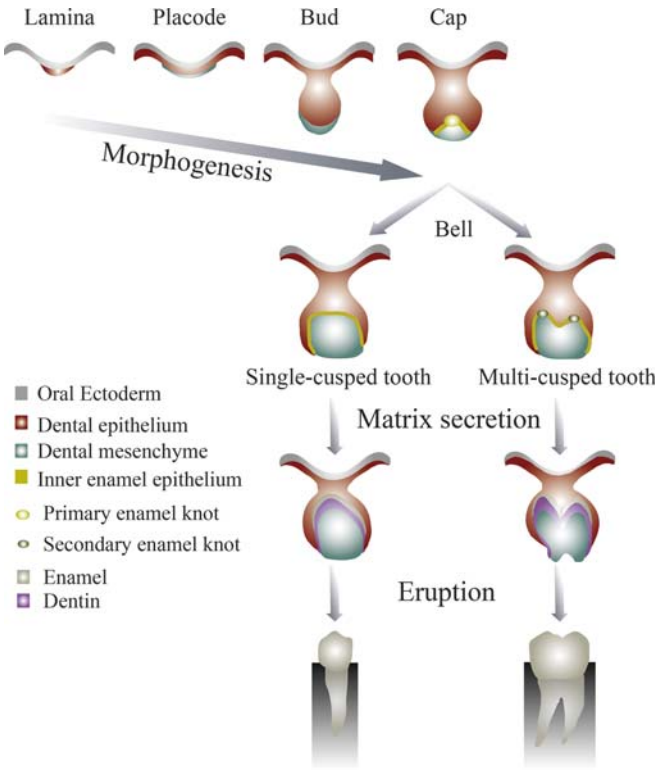

FIGURE 51.1 Principal stages of tooth formation [1].

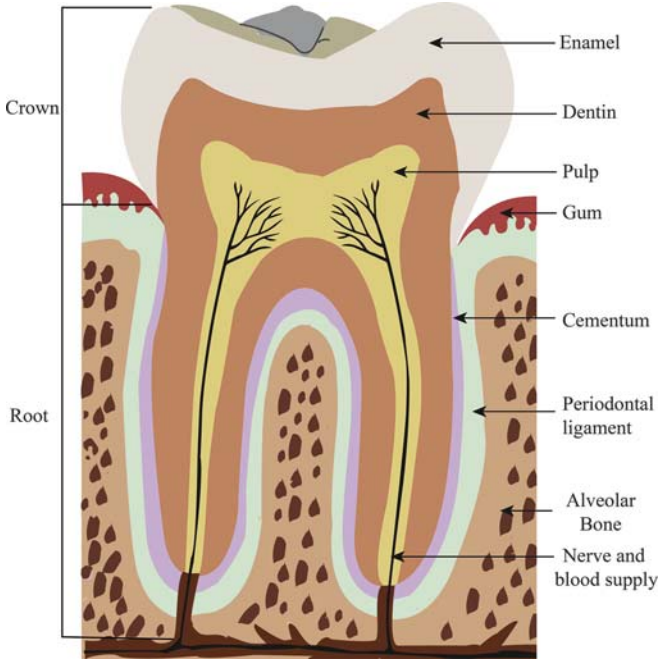

FIGURE 51.2 Adult human tooth morphology.

which secrete the enamel, largely undergo apoptosis before the tooth emerges into the oral cavity, with only a few dental epithelial cell remnants remaining in the periodontal ligament (PDL), termed the epithelial rests of Malassez. The tooth is attached to the jaw by specialized supporting tissues that consist of the PDL, the cementum, and the alveolar bone, all of which are derived from the neural crest cell–derived dental mesenchyme and which are protected by the gingiva (Fig. 51.2). To date, vital vascularized and innervated human adult teeth cannot be regenerated or regrown once damaged or lost. Therefore, the development of effective therapies to regenerate and repair lost or damaged teeth is a major goal of dental TE and regenerative medicine.

DENTAL STEM CELLS

DSCs can be collected from a variety of embryonic and postnatal or adult dental tissues. Embryonic stem cells (ESCs) are pluripotent stem cells that have the capacity to form all tissues of the body, including dental tissues [1,10]. ESCs can be obtained from the inner cell mass of a blastocyst in the 4- to 5-day-old embryo and from the embryonic germ in the 10- to 15-day-old embryo [10,16,17]. In contrast to ESCs, postnatal DSCs are multipotent stem cells that can develop into a restricted number of differentiated dental cell types [18]. The main functions of DSCs in natural teeth are to repair damaged dental tissues and maintain normal dental tissue turnover. Five different mesenchymal stem cell (MSC) populations have been identified in dental tissues, including dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), PDL stem cells (PDLSCs), dental follicle precursor cells (DFPCs), and stem cells from apical papilla (SCAPs). Each type of DSC population has specific characteristics and advantages for application in regenerative medicine and dentistry.

In 2000, Gronthos et al. were the first to report the isolation and identification of DPSCs in adult human dental pulp [19]. They showed that DPSCs can produce densely calcified nodules in in vitro tissue culture, but did not exhibit the capacity to form adipocytes, compared with bone marrow mesenchymal stem cells (BMSCs), which do. However, in 2002, Gronthos et al. reported the capacity of DPSCs to differentiate into both adipocytes and neural-like cells [20]. Periodontal ligament stem cells (PDLSCs) are found in PDL tissues located between the tooth and the alveolar bone and can be isolated from the roots of extracted teeth. PDLSCs have the capacity to differentiate into cementoblast-like cells, adipocytes, osteoblasts and collagen-forming cells [21]. Moreover, they were shown to regenerate a cementum–PDL-like structure and contribute to periodontal tissue repair [21]. SHEDs are isolated from an accessible tissue resource: autologous baby teeth [22]. They were identified as a population of highly proliferative, clonogenic cells capable of differentiating into a variety of cell types including neural cells,

adipocytes, and odontoblasts [22]. SHEDs showed the capacity to induce bone formation, generate dentin, and survive in mouse brain after in vivo transplantation. DFPCs can be isolated from the dental follicle, which surrounds the developing tooth and supports the formation of cementum, PDL, and alveolar bone [23]. DFSCs have the capacity to differentiate into osteoblasts, adipocytes, and nerve-like cells in in vitro culture [24–26]. DFPCs expressed higher amounts of insulin-like growth factor-2 transcripts than human BMSCs, and after in vivo transplantation in immunocompromised mice, DFPCs expressed osteocalcin (OCN) and bone sialoprotein (BS) but did not exhibit cementum or bone formation [23]. However, other studies showed that DFSCs transplanted into mice contributed to the formation of a new PDL and cementum tissues [27,28]. SCAPs can be isolated from the dental papilla, which normally develop into the tooth dentin and pulp. It was reported that SCAPs proliferate two- to threefold more rapidly than stem cells obtained from the pulp organ [29]. Also, SCAPs were demonstrated to differentiate into osteoblasts, odondoblasts and adipocytes in in vitro culture, and when implanted in vivo, they were shown to differentiate into osteoblasts and odontoblasts [30,31]. Immunophenotypically, SCAPs are similar to DPSCs with respect to osteogenic or dentinogenic and GF receptor gene profiles, and they also express the stem cell marker STRO-1 and dentinogenic markers including bone sialophosphoprotein, OCN, and the GFs fibroblast growth factor (FGF) recombinant 1 (FGFR1) and transforming growth factor (TGF)-β [29]. Moreover, upon stimulation with a neurogenic medium, SCAPs express a wide variety of neurogenic markers including nestin and neurofilament M.

Based on the promising characteristics of DSCs, dental TE efforts are focused on using DSC populations to bioengineer dental tissues, tooth supporting structures, and bioengineered whole teeth. Moreover, DSCs are considered to be a promising treatment method for several clinical conditions including Alzheimer disease, Parkinson disease, and spinal cord injury [32]. Here, we describe advances in dental TE to regenerate whole tooth and dental tissue structures, including dental pulp, dentin, PDL, and alveolar bone.

DENTAL TISSUE ENGINEERING

The basic principle of TE employs the use of cells, scaffolds, and bioactive agents to regenerate tissues similar to native human tissues (Fig. 51.3). In vivo delivery exposes cells to a host of survival challenges, including immune issues related to inflammation and autoimmunity [33]. To survive the initial onslaught of the immune response

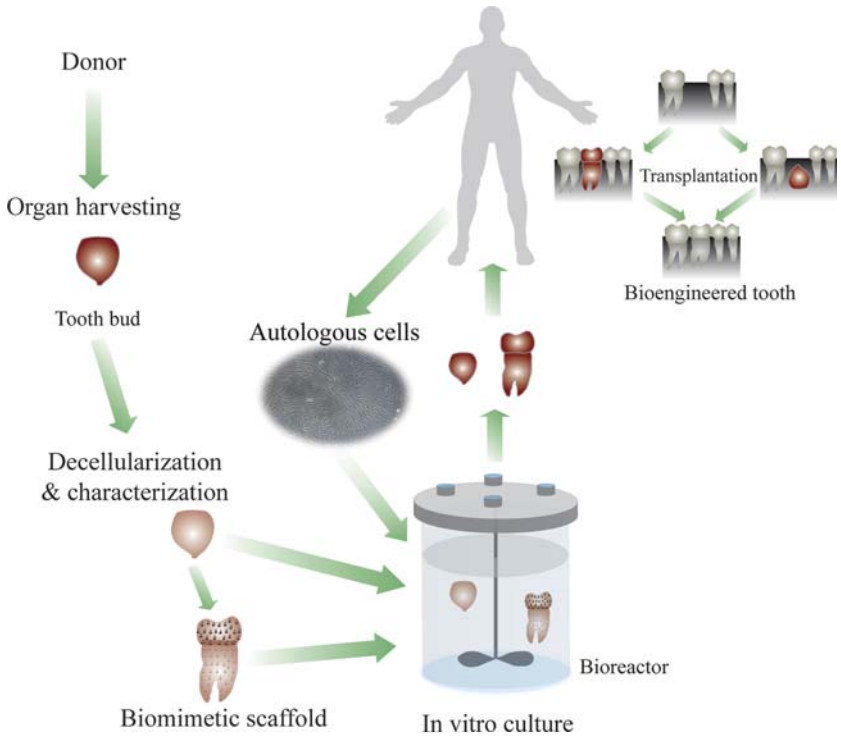

FIGURE 51.3 Dental tissue engineering approach [1].

better and avoid death, in vivo–delivered cells can be seeded in or onto a biodegradable scaffold before implantation when they are used to repopulate a tissue defect and/or restore function [34]. Ideal TE scaffolds mimic the properties and functions of the natural extracellular matrix (ECM), providing appropriate physical characteristics to support the development of new functional tissues. Therefore, it is important to consider the mechanical integrity and surface functionality of any given biomaterial scaffold, to ensure appropriate cell adhesion, proliferation, and differentiation [35,36]. Both natural and synthetic biomaterial scaffolds have been proposed for dental tissue regeneration [1,35,37–40]. The advantage of using natural scaffolds (i.e., collagen, alginate, fibrin, chitosan, gelatin, silk) is the ability to fine-tune their degradation rates by varying the concentration of the polymer and/or cross-linking agents. Synthetic scaffolds (polylactic acid, polyglycolic acid (PGA), PLLA, PLGA, PCL, etc.) have advantages including processing flexibility and the ability to be manufactured in any shape or size and with the desired predefined architecture and structural parameters [35]. Hydrogels are a specific class of scaffolds that exhibit huge potential for applications in dental tissue regeneration, owing to their versatility and adaptability [41,42]. Hydrogels offer several advantages including injectability, easy incorporation of therapeutic agents and cells under mild conditions, minimally invasive local delivery, and high contourability [42,43].

Bioactive agents such as GFs and nucleic acids have an important role in odontogenesis [12] and can be used to functionalize scaffolds to initiate the formation of new tissues [1]. As such, scaffolds can provide a multitude of advantages for bioactive agents, including safe delivery profiles, protection from biodegradation, and the ability to deliver the bioactive agents locally to where they are needed [13,44–46]. GF are critical to the development, maturation, maintenance, and repair of craniofacial and dental tissues [12]. It is therefore important to understand which of the GFs resident in the DSC niche provide appropriate cues to control their fate. For instance, GFs produced by DSCs during tooth development are responsible for the regenerative capacity of the dentin, which maintain cell proliferation and differentiation potential [12]. GFs function by binding to the extracellular domain of an appropriate target GF receptor, which in turn activates intracellular signal transduction pathways [47]. Several GFs (i.e., bone morphogenetic proteins [BMPs], sonic hedgehog [SHH], TGFs, FGFs, brain-derived neurotrophic factor [BDNF], and VEGF) are expressed during tooth formation and repair [1,15]. Therefore, dental tissue regeneration may be facilitated by incorporating GFs into scaffolds to promote dental cell differentiation. Also, because GF protein expression is the result of their gene expression [12], incorporating nucleic acid expression constructs into scaffolds has been proposed as a means to overcome certain limitations of GF delivery, including their short half-lives, denaturation during encapsulation processes, the time-consuming and expensive problem of GF production, the requirement for supraphysiological doses and GF combination for the most effective approach, the extended times required for cell differentiation, and difficulties in differentiating cells toward specific lineages [47–49]. Gene delivery of transcription factors can be used to ensure proper expression of particular splice variants in a coordinated time and sequence, and the ability to regulate a cascade of multiple genes, all from a single delivered construct [1]. Moreover, interference RNA (RNAi), a gene silencing mechanism, can be used to induce DSC differentiation [50].

Several approaches have been developed to incorporate a variety of bioactive agents into scaffolds and control their release profiles [45,46]. For instance, covalent immobilization of bioactive agents offers additional control over the spatiotemporal distribution of a particular agent compared with physical adsorption [1]. Immobilized bioactive agents can be released upon degradation of the matrix or by the hydrolysis of degradable links. Another way to entrap bioactive agents into scaffolds is by incorporating microparticles and nanoparticles [51–54]. The nanoscale properties of nanoparticles enable the ability to fine-tune release kinetics for improved transport properties, diffusivity, solubility, regulated biodistribution, minimization of toxic side effects, and the enhanced therapeutic index of bioactive agents. The combination of DSCs, biomaterial scaffolds, and bioactive agent delivery systems allows for the creation of multifunctionalized systems that can be used to facilitate dental tissue regeneration [1].

Notably, decellularized scaffolds and scaffold free approaches have created new areas of research in addition to the previously used natural and synthetic scaffold approaches [55–58]. Decellularized scaffolds, created by gently removing immunogenic cells from natural tissues such as heart, lung, bone, liver, and tooth buds, preserve the structure, shape compatibility, mechanical integrity, and bioactive molecule gradients that facilitate cell–cell interactions, cell adhesion, and ECM formation [55,59]. As such, detailed characterizations of ECM composition and organization in natural dental tissues could also facilitate dental TE efforts [56,60]. Finally, cell sheet technologies have been proposed to facilitate the regeneration of dental tissues including tooth root, pulp, dentin, and periodontal tissues [57,61–63]. This technique enables the creation of intact sheets of cells that can be harvested without trypsinization and preserves the ECM formed by the cells, including adhesive proteins such as fibronectin. Application of these TE approaches in dental tissue regeneration are discussed subsequently.

Whole Tooth Engineering

Several approaches to engineering entire biological teeth have been proposed, including dental TE, stimulation of third dentition formation, cell–tissue recombination, chimeric tooth TE, and gene-manipulated tooth regeneration [1]. The two major approaches used for tooth regeneration are cell–tissue recombination and dental TE approaches. Dental cell–tissue recombination approaches rely on replicating the natural processes of tooth development, in which cultured progenitor stem cell–tissue constructs are directly implanted in the defect site (Fig. 51.4). Many studies have reported the bioengineering of functional teeth from embryonic stem cells cultured in vitro and/or implanted in vivo [16,17,64–66]. Ohazama et al. showed that embryonic day (E)10 oral epithelium stimulated an odontogenic response in cultured neural and BMSCs [16]. Moreover, BMSC-derived recombinants were demonstrated to form tooth crown structures composed of enamel, dentin, and pulp. In contrast, tissue recombinants derived from ESCs and neural stem cells did not form teeth but expressed odontogenesis-related genes [16]. In another study, high cell–density suspensions generated from E14.5 incisor epithelial and mesenchymal tissues cultured in vitro or implanted into the subrenal kidney capsule subsequently formed bioengineered teeth when implanted in the extraction socket of a rat mandibular incisor [17]. These studies demonstrate the potential for using ESCs for tooth regeneration. However, the potential tumorigenic nature of transplanted ESCs, ethical issues regarding the use of human embryos, and the potential for allogeneic immune rejection are all major concerns of ESC-based therapies [10,67].

Adult DSCs, BMSCs, umbilical cord–derived MSCs, and induced pluripotent stem (iPS) all exhibit potential for use in dental TE [10,68]. Young et al. demonstrated the first successful use of adult DSCs seeded onto PGA–PLLA scaffolds to generate tooth crowns containing dentin and enamel; this demonstrated the presence of adult dental epithelial and MSC populations in pig third-molar tooth tissues [69]. Another study demonstrated that bioengineered pig tooth crowns containing dentin, pulp, and enamel formed in 25–30 weeks, whereas rat tooth crowns formed in just 12 weeks [70]. Honda et al. harvested dental cells from canine first-molar tooth buds, seeded them onto PGA scaffolds, and implanted them into tooth sockets of extracted teeth [71]. Subsequent analyses of harvested implants showed the formation of dentin and bone, but no enamel tissue or dental-root formation was observed. The same authors reported that dental epithelial and mesenchymal cells harvested from porcine third molar teeth seeded onto collagen scaffolds and implanted in vivo formed a single tooth in each scaffold that was morphologically similar to natural teeth [72]. In another interesting study, comparison of bioengineered dental tissues grown in the mandible versus the omentum revealed that both implant sites supported the formation of bioengineered dentin, enamel, pulp, and periodontal tissues [73]. However, omental implant dental tissues appeared to be more organized than those grown in the mandible. Yang et al. reported the formation of a complete tooth, consisting of a tooth crown, root, pulp, enamel, dentin, odontoblasts, cementum, blood vessels, and PDLs when tooth bud cells were suspended in fibrin glue and autografted back into the original alveolar sockets of a pig [74]. Smith et al. demonstrated the use of gelatin methacryloyl (GelMA) hydrogels, combined with postnatal porcine dental epithelial and dental mesenchymal progenitor cells, to support the formation of mineralized and functionally vascularized tissues of specified size and shape [75]. Also, the same model was improved by incorporating dental epithelial and dental mesenchymal cell sheets that expressed appropriate tooth marker expression patterns including SHH, BMP-2, Runt-related transcription factor 2, tenascin, and syndecan in vitro and in vivo [57]. Yang et al. reported whole tooth regeneration using DPSCs combined with epithelial cells isolated from gingival epithelium implanted into the mandibular alveolar socket of a pig for 13.5 months [66]. Seven of eight pigs developed two teeth containing crown, root, and pulp structures. Subsequent histological analyses demonstrated the formation of enamel-like tissues, dentin, cementum, odontoblasts, and periodontal tissues. All of the pig hosts formed regenerated molar teeth regardless

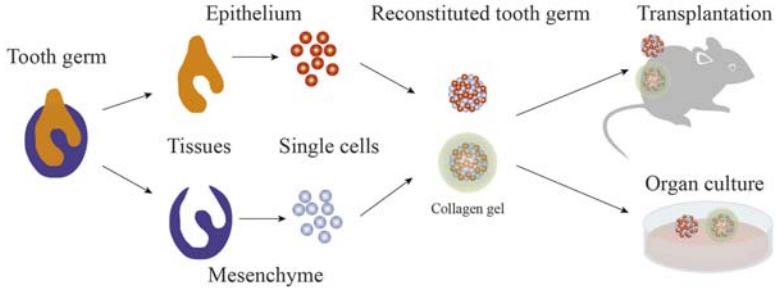

FIGURE 51.4 Dental cell tissue recombination approach [1].

of the original tooth type used to procure the DPSCs, whereas pig hosts that had tooth germs removed or received acellular scaffold implants did not develop new teeth. The authors also reported that the implant location may have influenced the morphology of the regenerated tooth.

More detailed characterizations of tooth expressed ECM molecules, including their respective developmental and spatial organization, may facilitate the design of effective scaffolds for tooth regeneration. One approach to achieving this goal included devising methods to decellularize and demineralize porcine molar tooth buds effectively while preserving natural ECM protein gradients [56]. This report showed that the collagen I, fibronectin, collagen IV, and laminin gradients present in natural tooth tissues were retained in decellularized tooth bud samples. Second harmonic−generation image analysis and three-dimensional (3D) reconstructions showed that natural tooth tissue exhibited higher collagen fiber density and more organized collagen fibers compared with decellularized tooth tissue. That report showed that dental cells seeded back into the decellularized tooth bud scaffolds were able to establish residence within the scaffold and to elaborate and remodel the matrix. Ongoing research has focused on detailed 3D characterizations of tooth pulp tissue to examine collagen fiber destruction and remodeling as a consequence of the decellularization and reseeding processes.

Dental Pulp and Dentin Regeneration

Dental pulp consists of DPSCs, odontoblasts, endothelial cells, neurons, immune system cells, GFs and the ECM, all of which are crucial for maintaining the functions of healthy teeth. Dentin, which encases the dental pulp, is a mineralized form of the collagen-based predentin matrix; its crystalline structure primarily consists of HA and water [76]. Creating an in vitro tissue model that takes into account all of the aspects of a pulp−dentin microenvironment is challenging. Nonetheless, adopting a deconstruction strategy to reduce the pulp−dentin ecosystem to a few of the main components anticipated to be involved in maintaining functional pulp−dentin biology may be sufficient to establish in vitro models capable of pulp−dentin regeneration. Because interactions between tissue vasculature and nerves is critical to maintaining dental pulp homeostasis, dental TE strategies for pulp regeneration must recreate a microenvironment that supports the cellular cross-talk needed to maintain the healthy, functional tissue heterogeneity of natural tooth pulp [76]. Advances in the field of TE have made dental pulp regeneration a realistic, attractive, and alternative therapy for endodontic treatments to restore damaged teeth [5,6,77−81]. The goal is to replace the damaged pulp−dentin complex with a bioengineered, functional biological tissue surrogate that can integrate with remaining healthy host tissues. The first evidence of novo pulp regeneration was demonstrated using human tooth slices or root segments (RSs) injected with DSCs combined with scaffolds [79]. In that report, Cordeiro et al. showed that SHED cell-seeded PLLA scaffolds placed within human tooth slices and transplanted into immunodeficient mice created bioengineered tissue architecture and cellularity closely resembling those of physiologic dental pulp [79,81], including SHED cell differentiation into odontoblast and endothelial-like cells. When supplemented with VEGF, SHED cells expressed VEGF receptor2, CD31, and vascular endothelial cadherin and organized into capillary-like sprouts [81]. These studies demonstrated that the VEGF signaling pathway is an important regulator of endothelial cell−controlled differentiation of DSCs, and therefore that scaffold biomaterials could be used to control the delivery of GFs such as VEGF for dental TE [13]. Another study consisted of SCAPs and DPSCs seeded onto PLG scaffolds, inserted into tooth RSs and transplanted in mice. Analyses of harvested implants showed that the root canal space was filled entirely by a pulp-like tissue exhibiting well-established vascularity, and the formation of a continuous layer of dentin-like tissue deposited along the dental wall of the tooth root canal. The newly formed dentin like tissue was elaborated by a layer of newly formed odontoblast-like cells expressing dentin sialophosphoprotein, BS, alkaline phosphatase, and CD105.

For optimized clinical translation, dental pulp regeneration will require the use of injectable scaffolds. Rosa et al. tested the hypothesis whether SHED encapsulated in PuraMatrix hydrogel or collagen I, could regenerate a functional dental pulp when injected into full-length root canals and implanted in vivo [77]. The authors reported the formation of pulp-like tissues, including the presence of odontoblasts capable of generating new tubular dentin throughout the root canals. The engineered pulp tissue exhibited cellularity and vascularization similar to those of natural human dental pulps. Therefore, this strategy might successfully facilitate the completion of tooth root formation in damaged, necrotic, immature permanent teeth. In another study, autologous DPSCs encapsulated in collagen were transplanted into a root canal along with stromal cell−derived factor-1 (SDF-1) after pulpectomy of mature teeth with complete apical closure in dogs [82]. The results showed that by day 14, the root canal was successfully filled with regenerated pulp tissue, including nerves and vasculature, followed by new dentin formation along the dentinal wall. Khayat et al. also defined a reliable method to regenerate pulp-like tissues within tooth RSs using 5% GelMA hydrogel-encapsulated human DPSCs−human umbilical vein endothelial cells

(HUVECs) implanted subcutaneously in nude rats for 4 and 8 weeks [6]. Analyses of harvested implants showed that hDPSC−HUVEC-encapsulated GelMA constructs formed pulp-like tissue that adhered to the inner dentin surface of the RS, including odontoblast-like cells exhibiting cell extensions into the dentin tubules. Moreover, that report showed that GelMA hydrogels supported hDPSC−HUVEC cell attachment, proliferation, and infiltration of host cells and promoted the establishment of well-organized neovasculature formation. This study identified GelMA hydrogels combined with DSCs as a promising, clinically relevant pulpal revascularization treatment to regenerate human dental pulp tissues. Another study showed that SHEDs and DPSCs cultured in peptide−amphiphile hydrogel scaffolds exhibited cell proliferation and differentiation within the scaffolds, and that SHEDs formed a soft tissue whereas DPSCs deposited mineral [43]. These hydrogel scaffolds are desirable for clinical applications for pulp regeneration, because they are easy to handle and can be introduced into small defects or root canal. Hyaluronic acid sponge scaffolds also exhibit an appropriate structure, biocompatibility, and biodegradation for dental pulp regeneration [83]. In vivo studies using hyaluronic acid−based scaffolds in an amputated dental pulp of rat molar showed dental pulp proliferation and blood vessel invasion. Prescott et al. investigated the role of DPSCs, collagen scaffold, and dentin matrix protein 1 (DMP1) in a simulated furcal perforation, in vivo mouse model [84]. The successful formation of organized pulp tissue was observed in the group containing the triad of DPSCs, a collagen scaffold, and DMP1. In another report, a chitosan bilayer membrane containing TGF-β1 releasing microspheres was developed to promote reparative dentin formation in a pulp-capping dog model [85]. Analyses of in vivo implanted constructs showed that TGF-β1−releasing chitosan membranes generated reparative dentin three to six times thicker than those generated by chitosan bilayer membrane alone. In another study, decellularized human dental pulp ECM supported the proliferation and differentiation of SCAP into odontoblast-like cells near pulp chamber dentinal walls [86]. That study demonstrated that human dental pulp from healthy extracted teeth can be successfully decellularized and subsequently used to differentiate reseeded DSCs, potentially improving clinical outcomes and ultimately promoting the survival and function of injured teeth. Ravindran et al. also demonstrated the odontogenic differentiation of both human DPSCs and human PDLSCs when cultured on a decellularized 3D pulp ECM scaffold, without the need for the exogenous addition of GFs. Subcutaneous implantation of ECM scaffolds containing DPSCs showed the formation of dental pulp-like tissue containing cells expressing DSP and dentin phosphophorin . These results showed that a decellularized dental pulp ECM scaffold can be used as a biomimetic scaffold for dental tissue regeneration and as tool to study the extracellular function of multifunctional proteins [60].

Kim et al. reported the regeneration of dental pulp-like tissue in human teeth by cell homing and without cell transplantation [80]. Human teeth implanted in the mouse dorsum and delivering basic FGF (bFGF) and/or VEGF formed recellularized and revascularized connective tissue that integrated with native dentinal walls of tooth root canals. Also, the combined delivery of bFGF and VEGF, or platelet-derived growth factor (PDGF) combined with a basal set of nerve growth factor and BMP-7, generated cellularized and vascularized tissues and neodentin formation over the surface of native dentinal walls. In another study, scaffold-free 3D tissues were engineered from DPSC sheets, placed into the canal space of human tooth RSs, and implanted subcutaneously into mice for pulp regeneration [87]. Histological results indicated that after 3−5 months, implanted tooth roots containing 3D scaffold-free engineered tissues exhibited vascularized fibrous tissue formation throughout, whereas empty tooth roots remained predominantly empty.

Periodontal Regeneration

Periodontal tissue regeneration strategies aim to restore the supporting periodontal tissues of teeth, including the formation of new cementum, PDL, and alveolar bone [88]. Clinical studies show that transplantation of autologous PDL progenitor (PDLP) cells may be a useful therapy to repair periodontal defects [89]. It was shown that PDLPs were similar to PDLSCs with respect to their ability to exhibit high proliferation rates, express mesenchymal surface molecules, and regenerate in vivo tissue; this provides clinical and experimental evidence supporting the efficacy and safety of using autologous PDL cells in treating human periodontitis. In another study, human PDLSCs were transplanted into immunocompromised mice and rats to assess their capacity for tissue regeneration and periodontal repair [21]. Using defined in vitro culture conditions, PDLSCs were shown to differentiate into cementoblast-like cells, adipocytes, and collagen-forming cells. When transplanted into immunocompromised rodents, PDLSCs exhibited the capacity to generate cementum−PDL-like tissues and contribute to periodontal tissue repair. Therefore, transplantation of PDLSCs may hold promise as a therapeutic approach for reconstructing tissues destroyed by periodontal diseases. It was also reported that autologous PDLSCs, combined

with HA−β-TCP scaffolds and transplanted into surgically created periodontal defects in pigs, were capable of regenerating periodontal tissues, leading to a favorable treatment for periodontitis [90]. Using a similar approach, it was shown that transplanted human SCAPs and PDLSCs, combined with HA−TCP ceramic particles and implanted in an in vivo swine model, regenerated a functional tooth root and periodontal complex capable of supporting a porcelain crown [91]. As such, this approach led to the recovery of both tooth strength and appearance.

Zhou et al. reported a PDL cell-sheet delivery system to promote periodontal tissue healing in a canine model [62]. After root canal treatment, the tooth roots were wrapped with PDL fibroblast-derived cell sheets and replanted back into the same tooth socket for 8 weeks. Subsequent analyses of the in vivo implanted teeth showed that multilayered PDL cell sheet constructs attached to the tooth root and that most cells of the PDL sheet-tooth constructs were viable after replantation. The PDL cell-sheet group showed a significantly higher occurrence of healing (88.4%) than the control group without cell sheets (5.3%). PDL and cementum tissue regeneration was observed in the experimental group and the regenerated tissues expressed high levels of collagen type III, collagen type I, and fibronectin expression. In a similar study, three-layered PDL cell sheets supported by PGA nanofibers were transplanted to dental root surfaces exhibiting a three-wall periodontal defect while filling existing bone defects with porous β-TCP. PDL cell sheet transplantation resulted in regenerated new bone formation as well as the formation of cementum containing embedded, properly oriented collagen fibers. These results suggested that PDL cells exhibit the ability to differentiate into periodontal tissues composed of both hard and soft PDL tissues, and that PDL cell sheet transplantation may be useful for periodontal regeneration in clinical settings [92]. In another study, Lei et al. characterized the cell properties of DPSCs and PDLSCs after in vivo implantation [93], showing that DPSCs and PDLSCs can maintain MSC-like characteristics after in vivo implantation compared with PDLSCs, DPSCs appear to be much more stable under in vivo conditions. These findings provide additional cellular evidence supporting and expanding the use of dental tissue−derived stem cells in dental TE.

In another study, intrabony defects were created in rats to evaluate the regenerative potential of an injectable macroporous calcium phosphate cement (CaP) combined with BMP-2 or FGF-2. Results from that study showed that the combined topical application of FGF-2 with an injectable CaP may prove to be a promising treatment for periodontal regeneration [94]. Another study, by Kim et al., tested the hypothesis that anatomically correct teeth can be regenerated in scaffolds without the need for cell transplantation. Anatomically shaped human molar and rat incisor scaffolds were fabricated by 3D bioprinting of hybrid PCL−HA with 200-μm-diameter interconnecting microchannels. SDF1 and BMP-7 were delivered into the microchannels of the scaffold, which were then implanted orthotopically after mandibular incisor extraction in the case of rats, or by using human molar-shaped scaffolds implanted ectopically into the rat dorsum. Analyses of explanted constructs showed the regeneration of PDL and new bone, which formed at the interface of the rat incisor scaffold and the native alveolar bone. This study showed that SDF1 and BMP-7 delivery recruited significantly more endogenous cells and also induced a greater angiogenesis response compared with growth factor−free control scaffolds [95]. Porous chitosan−coral composites combined with plasmid-encoding PDGF-B gene were also tested for periodontal regeneration, showing that subcutaneous implantation of gene-activated scaffolds supported greater cell proliferation than pure coral scaffolds, and that PDGF-B and type-I collagen expression was upregulated in gene-activated scaffold. Therefore, coral scaffolds combined with PDGF-B gene delivery may also serve as a suitable approach for periodontal tissue regeneration [96]. In another study, a mesoporous bioglass−silk fibrin scaffold combined with BMP-7 and/or PDGF-B adenovirus synergistically promoted up to twofold greater regeneration of PDL, alveolar bone, and cementum compared with each adenovirus used alone [97]. Ex vivo BMP-7 gene transfer was used to stimulate the repair of large mandibular alveolar bone defects in a rat wound repair model [98], consisting of syngeneic dermal fibroblasts transduced ex vivo with adenoviruses encoding green fluorescent protein, BMP-7 (Ad-BMP-7), or an antagonist of BMP bioactivity, noggin (Ad-noggin). These studies demonstrated that Ad-noggin treatment inhibited osteogenesis compared with the control- and Ad-BMP-7−treated specimens. Furthermore, the osseous lesions treated with Ad-BMP-7 gene delivery demonstrated rapid chondrogenesis and subsequent osteogenesis, cementogenesis, and predictable bridging of the periodontal bone defects [98]. In another study, a thermosensitive chitosan hydrogel was used as a small interfering RNA (siRNA) reservoir to silence receptor activator of nuclear factor-κB signaling and promote PDL regeneration [50]. The cumulative in vitro release of siRNA from the hydrogel was 50% over 14 days, and high PDL cell viability was observed for cells seeded on the siRNA-loaded scaffold. In vivo studies showed that the fluorescent signal from siRNA within hydrogel was maintained for up to 14 days when subcutaneously implanted in mice [50].

Alveolar Bone Regeneration

Jaw reconstruction can be challenging because both teeth and bone must be restored [99]. Autologous bone graft techniques followed by the placement of dental implants is one approaches being used to repair jaw defects. However, undesirable limitations to this approach include associated donor-site morbidity for harvested bone, insufficient quantities of available bone, and difficulties in dental implant placement owing to inadequate size, shape, and quality of the reconstructed alveolar ridge [100,101]. Because of high rates of progressive periodontitis, which can alter alveolar bone morphology and destroy surrounding tooth-supporting tissues, alveolar bone is highly susceptible to inflammation, which may result in necessary tooth extraction. The alveolar ridge may continue to resorb even after dental implant placement, and dysregulated bone remodeling in response to mechanical loading (mastication forces) may occur owing to uneven strain distributions caused by resorbed alveolar bone tissues [101]. Therefore, reliable tooth and alveolar bone regeneration strategies are needed to repair jaw defects effectively.

Inorganic materials such as CaP ceramics, bioactive glass, and ceramic−polymer composites have been especially developed for bone TE applications [39,101]. HA ceramics, β-TCP cements, and biphasic calcium phosphates are examples of synthetic CaP bone substitutes [101]. Although they lack the mechanical properties of naturally formed bone, they exhibit osteoinductive or osteogenic abilities, and these ceramics gradually acquire mechanical strength similar to that of cancellous bone [101,102]. For alveolar bone regeneration, biodegradable granular forms of ceramics are preferred, such as β-TCP, because they are easy to shape and can easily adopt the 3D size and shape of the bony defect, an important consideration for proper aesthetics [102]. Advantages of combining ceramics with polymers include improved biodegradability, biocompatibility, and an ability to readily bind GFs critical for osteoinduction. PCL scaffolds coated with CaP or HA exhibit improved osteoblast adhesion, spreading, and proliferation and promote alveolar bone formation in periodontal defects [101]. Goh et al. used a monkey model to examine periimplant bone regeneration and implant stability after immediate implant placement into tooth sockets containing facial wall defects [103]. In the control group, the bony defect was reconstructed with autogenous particulate bone, whereas in the test group, PCL−TCP scaffolds were used. Histological analyses showed better maintenance of facial bone contour in the test group; however, bone regeneration was observed only in areas adjacent to the bony wall of the defect. Implant survival was 100% 6 months for both groups, but the use of a PCL−TCP scaffold showed better maintenance of the alveolar contour compared with autogenous particulate bone at 6 months, although there was minimal bone regeneration within the defect [103].

Aquino et al. reported the use of autologous human DSPCs combined with a collagen sponge scaffold for oral maxillofacial bone tissue repair in patients requiring third-molar extraction [104]. Three months after autologous DSPC grafting, they reported that patient alveolar bone showed optimal vertical repair and complete restoration of periodontal tissue. Moreover, optimal bone regeneration was still evident 1 year after grafting. This clinical study demonstrated the effectiveness of DSCs combined with scaffolds to restore dental tissues such as alveolar bone and periodontal tissues [104]. In another study, bone implants created from pig iliac crest−derived MSCs seeded onto lattice scaffolds and grown in a rotational oxygen-permeable bioreactor system (ROBS) reactor were then combined with dental epithelial and dental mesenchymal cell−derived tooth constructs created from unerupted tooth buds harvested from the same pig [105]. Analyses of harvested implants revealed the formation of bioengineered mineralized tooth tissues, including primary and reparative dentin and enamel in the tooth portion of the hybrid tooth−bone implants, and the formation of OCN and BS-positive mineralized bone in the bone portion of the hybrid tooth−bone constructs. Collagen type III−positive connective tissue resembling PDL and tooth root structures were also present at the interface of the bioengineered tooth and bone tissues. These results demonstrate the potential use of hybrid tooth−alveolar bone constructs for clinical treatment of tooth loss accompanied by alveolar bone resorption [105]. In a similar study, tooth−bone constructs were prepared from third-molar tooth tissue and iliac crest bone marrow−derived osteoblasts isolated from, and implanted back into, the same pig as an autologous reconstruction [100]. Subsequent analyses showed the formation of small tooth structures consisting of organized dentin, enamel, pulp, cementum, and PDL surrounded by newly formed bone, indicating the possibility of tooth regeneration and associated alveolar bone in a single procedure [106].

Cai et al. studied which differentiation approach (e.g., maintenance of stemness, osteogenic or chondrogenic induction) was most suitable for periodontal regeneration, using in vivo implanted, rat BMSC-seeded PLGA−PCL electrospun scaffolds. Their results showed that BMSCs exhibited chondrogenic differentiation, followed by regeneration of alveolar bone and ligament tissues [107]. Successful regeneration of alveolar bone and surrounding

periodontal tissues using gene therapy vectors such as adenoviral BMP-7 (Ad-BMP-7) was also achieved in vivo [98]. Mesoporous Bioglass−silk fibrin scaffolds combined with BMP-7 and/or PDGF-B adenovirus synergistically promoted periodontal regeneration by enabling up to twofold greater regeneration of PDL, alveolar bone, and cementum tissues compared with each adenovirus used alone [97].

CONCLUSIONS

Root canal therapy and prosthetic dental procedures such as dental implants are the only clinical treatments available to treat necrotic dental pulp tissue defects and tooth replacement. These treatments present numerous disadvantages, including loss of tooth vitality and the inability to mimic properties and functions of natural teeth. Based on the promising characteristics of DSCs for regenerative tissue applications, dental TE efforts are focused on using these stem cell populations to bioengineer tooth supporting tissues and whole tooth. Although a variety of studies support the potential use of ESCs for tooth regeneration, the possibility of malignant tumorigenesis, ethical issues regarding the use of embryos, and the potential for allogeneic immune rejection make this approach problematic and unrealistic at present. In contrast, adult DSCs and iPS cells both exhibit significant potential for utility in dental TE. Successful use of DSCs for applications in dental tissue regeneration requires the combination of appropriate scaffolds and GF signals to induce dental cell differentiation and dental tissue formation. In addition, the concepts of scaffold-free approaches and cell homing have also been developed, suggesting alternative approaches using dental cell sheets and the recruitment of endogenous MSCs for dental tissue by GFs.

These reports demonstrate that it is possible to regenerate correct teeth anatomically using a combination of dental cells, scaffolds, and signals. Indeed, the tooth organ is a highly complex biological organ whose formation requires the intricate regulation of a cascade of molecular signals and gene expression. As such, a better understanding of the biological processes and interactions of important GFs and gene expression patterns regulating natural tooth development is critical for successful dental tissue regeneration. Methods to differentiate DSCs successfully into tooth specific lineages, to create all of the tissues that comprise a functional living tooth, is a main challenge in DSC research. New and effective methods for delivering bioactive agents such as GFs and nucleotides (i.e., plasmid DNA and RNA interference) combined with optimized biomaterials scaffolds, are anticipated to provide the means for successful alveolar bone and tooth regeneration in the foreseeable future, to meet the challenge of generating functional, living, bioengineered dental tissues.

List of Abbreviations

BMP Bone morphogenetic protein
BS Bone sialoprotein
CaP Calcium phosphate cement
DFPC Dental follicle precursor cells
DPSC Dental pulp stem cells
ECM Extracellular matrix
ESC Embryonic stem cells
FGF Fibroblast growth factor
GF Growth factors
HA Hydroxyapatite
OCN Osteocalcin
PCL Polycaprolactone
PDGF Platelet-derived growth factor
PDL Periodontal ligament
PDLSC Periodontal ligament stem cells
PGA Polyglycolic acid
PLGA Poly(lactic-*co*-glycolic acid)
PLLA Poly-L-lactide
SCAP Stem cells from apical papilla
SHED Stem cells from human exfoliated deciduous teeth
TCP Tricalcium phosphate
TGF Transforming growth factors
VEGF Vascular endothelial growth factor

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CHAPTER

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Cell Therapy for Blood Substitutes

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INTRODUCTION

RBCs, the oxygen-carrying component of the blood, are transfused in over half of all anemic patients admitted to intensive care units in the United States [1–3]. It is estimated that nearly 5 million patients receive approximately 14 million units of RBCs per year in the United States alone [4,5]. Limitations in the supply of RBCs can have potentially life-threatening consequences for patients, specifically for those who have rare or unusual blood types with massive blood loss caused by trauma or other emergency situations. Unfortunately, the supply of transfusable RBCs, especially the "universal" donor type (O)Rh-negative, is often insufficient, particularly in the battlefield environment and/or major natural disasters owing to the lack of blood type information and the limited time required for lifesaving transfusion. Moreover, the low prevalence of (O)Rh-negative blood type in the general population (<8% in Western countries and <0.3% in Asia) further intensifies the consequences of blood shortages for emergency situations in which blood typing may not be possible.

Platelets, anucleate discoid-shaped cell fragments released from megakaryocytes (MK), are essential to hemostasis, the biological process by which bleeding stops. Platelets adhere to damaged blood vessels and trigger a series of biochemical changes that stimulate clot formation and vascular repair. In cases of thrombocytopenia (platelet counts $< 150 \times 10^3/\mu L$), the increased risk of bleeding can have life-threatening consequences [6]. In the United States, approximately 1.5 million platelet transfusions are performed annually to protect patients, including those treated with chemotherapy or stem cell transplantations, from the risk of thrombocytopenia and its related dangers [7]. Unfortunately, refractoriness occurs in approximately one out of every three patients who require repeated platelet transfusions [8,9] and whereas both immunological and nonimmunological complications may be to blame, HLA alloimmunization is the primary cause of refractoriness [8]. Over the past few decades, the steady increase in demand for platelets combined with their limited shelf life has presented a constant challenge for blood centers and donor-dependent programs. As with RBCs, there is a vast and continuous need for functional, transfusable platelets, especially in times of emergency.

HSCs located within the bone marrow are multipotent stem cells discovered more than half century ago by Till and McCullogh [10] that have the ability to self-renew indefinitely and are responsible for replenishing all mature cells within the adult blood system including RBCs, platelets and myeloid and lymphoid cells. More than 30,000 patients with malignant blood diseases after chemotherapy received HSC transplantation; however, difficulty in finding an HLA-matched HSC donor, especially for individuals from a mixed or minority ethnicity, is the major obstacle to HSC regenerative therapy.

PSCs, including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), are capable of expanding indefinitely in culture without losing their pluripotency; this makes them an attractive cell source to be used for the large-scale production of a variety of therapeutic cell types [11–13]. The advent of human iPSCs may allow patient-specific therapies to be produced, thus circumventing potential issues with HLA mismatching and immunoincompatibility. Although each has its own advantages and disadvantages, hESCs and iPSCs represent two types of PSCs that may be able to serve as an alternative source for producing transfusable RBCs and platelets,

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and HSCs with long-term engraftment capability. Here, we will review progress in developing these lifesaving blood substitutes, including some of our own efforts in unlocking the potential use of hESCs and iPSCs in these endeavors.

RED BLOOD CELLS

Erythropoiesis

Erythropoiesis is a highly regulated, multistep process by which the body generates mature RBCs or erythrocytes. During mammalian development, erythropoiesis consists of two major waves: (1) primitive erythropoiesis, which is initiated in the yolk sac with the generation of large *nucleated* erythroblasts, and (2) definitive erythropoiesis, which arises from the fetal liver with the development of smaller *enucleated* erythrocytes [14]. Definitive erythropoiesis in fetal liver features the production of enucleated RBCs that quickly become dominant in embryonic circulation. The switch of hemoglobin to fetal types ($\alpha_2\gamma_2$) also occurs at the initiation of definitive erythropoiesis [15–17]. However, reports show that yolk sac–derived primitive erythroblasts can also enucleate in the circulation of a mouse embryo and persist throughout gestation [18,19].

In adults, all blood cell types, including lymphocytes, myeloid cells, and RBCs, are derived from HSCs residing in the bone marrow. The initial differentiation of a multipotential HSC into a common myeloid progenitor (CMP) determines its capacity to differentiate further into granulocytes, erythrocytes, megakaryocytes, and macrophages, but not lymphoid cells. As the CMP continues to differentiate, it undergoes significant expansion and will eventually commit to one particular lineage. Erythroid unilineage commitment leads to the appearance of the pronormoblast (also called the proerythroblast or rubriblast). The pronormoblast will then pass through early, intermediate, and late normoblast (erythroblast) stages before expelling its nucleus and becoming a reticulocyte. Upon exiting the bone marrow, reticulocytes enter the blood circulation and become fully mature RBCs, expressing adult forms of hemoglobin ($\alpha 2\beta 2$) and delivering oxygen to tissues of the body. They circulate for about 120 days before they are engulfed by macrophages and recycled [20]. The various stages of erythropoiesis can also be distinguished by characteristic morphological features in the cell cytoplasm and nucleus, which become evident after Wright–Giemsa staining. In addition, using an in vitro colony-forming assay, CMP progenitors can be identified by their ability to form a characteristic colony-forming unit (CFU), called the CFU–granulocyte, erythroid, macrophage, megakaryocyte (GEMM) whereas early erythroid progenitors develop into burst-forming units–erythroid (BFU-E), and late erythroid progenitors become CFU-erythroid (CFU-E) in this assay [14].

"Universal" Blood Generated by Modifying Red Blood Cell Surface Antigens

Dr. Lansteiner's Nobel Prize–winning discovery of ABO blood groups occurred over a century ago. Now 30 known human blood group systems and complicated issues surrounding blood type incompatibility continue to frustrate clinicians and scientists. To circumvent these issues, researchers have been trying to develop "universal" blood for decades, primarily through chemical modifications on the surface of RBCs. Among these efforts, Goldstein and coworkers demonstrated that group B erythrocytes can be enzymatically converted to group O, and that the converted cells survived normally in A, B, and O individuals [21]. Since this groundbreaking discovery, there have been coordinated efforts to identify both clinically and economically viable alternative enzymes. New classes of bacterial exoglycosidases have been discovered that can enzymatically perform group O conversions with faster kinetics [22,23]; yet the likelihood of this technology having a major role in blood transfusion practice is unclear. There are no reports showing the enzymatic conversion of other important blood typing groups such as the Rh factor. With the momentum of the rapidly advancing stem cell field, the concept of (O)Rh- negative RBCs derived from PSCs clearly offers an attractive option for the future of blood transfusions.

Red Blood Cells Generated From Adult Stem Cells In Vitro

Erythrocytes have been derived from a variety of primary stem cell sources including umbilical cord blood (CB), peripheral blood (PB), and bone marrow (BM). $CD34^+$ cells from CB, PB, and BM have been isolated and differentiated into erythrocytes with 95% purity after a little over a week of culture using erythropoietin (EPO), stem cell factor (SCF), and interleukin-3 [24–26]. Coculturing with a mouse MS-5 stromal cell line or human mesenchymal stem cells facilitates enucleation, the hallmark of mature RBCs. The stem cell–derived erythrocytes have properties similar to normal RBCs, including membrane deformation capacity, intrinsic enzymatic activity, and balanced adult

or fetal forms of hemoglobin that can bind and release oxygen. In addition, these erythrocytes have been found to survive in vivo in NOD/SCID mice and were detectable 3 days after transplantation. Douay and colleagues showed that autologous RBCs generated under good manufacturing practices (GMP) conditions from PB $CD34^+$ cells behaved the same as did their natural counterparts when reinfused into the autologous recipient [27]. This first preclinical study provided proof of principle for transfusion of in vitro–generated RBCs and the necessary safety data that could support an investigational new drug application. Stem cells from PB and BM have limited expansion capacity (29,000- and 16,500-fold, respectively) compared with those from CB (140,000-fold). Yet, cells from adult PB and BM are often easier to obtain and display mature forms of hemoglobin, whereas CB sources are more difficult to obtain and cells derived from them express only fetal globins. Despite their potential utility and substantial in vitro expansion using bioreactors [28], however, these primary cells still represent donor-limited sources of blood substitutes.

Red Blood Cells Generated From Human Embryonic Stem Cells

The expansion capacity of hESCs far exceeds that of BM, PB, or even CB. Hematopoietic precursors as well as more mature progeny representing erythroid, granulocyte, macrophage, megakaryocytic, and lymphoid lineages have all been identified in differentiating hESC cultures [29]. Therefore, many groups have focused their efforts on trying to steer the in vitro differentiation of entire hESC cultures into specific blood cell types such as RBCs or megakaryocytes and platelets [30–36].

The controlled differentiation of hESCs into erythrocytes has primarily been achieved by either embryoid body (EB) formation or coculturing with stromal cells followed by the isolation of $CD34^+$ cells and further expansion or differentiation. Although somewhat different in approach, both systems have encountered the same obstacles in generating fully mature adult RBCs. For example, Chang et al. [37] generated erythroid cells from hESCs by isolating and expanding nonadherent cells of day 14 EBs for an additional 15–56 days. The resulting cells coexpressed high levels of embryonic ε- and fetal γ-globins but few or no adult β-globins. In addition, the cells had not enucleated. Using a stroma coculture method, erythroid cells could be generated by cultured with human fetal liver stromal cells (FHB-hTERT) for 14–35 days, isolating $CD34^+$ cells and further differentiating them in a four-step culture system. In steps 1 and 2, cocktails of cytokines were used to promote the proliferation and maturation of erythroid precursors. In steps 3 and 4, erythroid cells were transferred onto mouse BM stromal cells (MS5) to facilitate terminal maturation [38]. Although these erythrocytes were generated on a relatively large scale ($0.5–5 \times 10^7$ cells), the resulting cells had problems similar to those generated by the EB method: they mainly expressed embryonic ε- and fetal γ-globin isoforms, with only a trace amount of adult β-globin being detected. Despite these reports, other studies suggest that specific types of stroma can in fact facilitate the expression of adult β-globin in developing erythrocytes. Immunostaining with globin chain-specific monoclonal antibodies [39] showed that almost 100% of hESC-derived erythrocytes expressed the adult β-globin chain after coculture with murine fetal liver derived stromal cells; however, the β-globin content and identity were not verified by an accurate physical method such as high-performance liquid chromatography or mass spectrography. Yet, most of the cells still had not enucleated, a problem that does not seem to have an easily explainable mechanism or simple solution. For example, a study published in 2005 showed that coculturing $CD34^+$ CB, PB, and BM cells on MS5 stroma produced RBCs with up to 100% enucleation [24], whereas a year later, Miharada et al. [26], reported that up to 77% of CB-derived erythrocytes could achieve enucleation without the use of stromal cells.

The mechanism(s) by which stromal cells may facilitate erythrocyte enucleation are largely unknown; therefore, it remains to be determined whether stroma coculture will be absolutely required for the enucleation of hESC-derived erythrocytes. Stroma may secrete important soluble factors, provide critical cell–cell contact, and/or engulf of nuclei and other organelles. Some studies suggest that the process of enucleation involves a critical asymmetric cell division [40] and that Rac GTPases and their effector, mDia2, extrude the pyknotic nucleus during this process [41]. Other studies show that in vivo, contact with macrophages is important and that the erythroblast-macrophage-protein Emp has a critical role in enucleation [42–44]. Clearly, further studies will be required to determine how to improve the efficiency of both enucleation and globin switching in vitro. Coculture with stroma may circumvent problems with the processes, but production of clinical-grade RBC substitutes will demand stroma-free culture conditions.

Not only must in vitro–generated RBC substitutes be fully matured and enucleated for use in the clinic, they must also be capable of large-scale production. Related to this issue, we have developed a strategy that generates functional hemangioblasts (the common precursor cell to all hematopoietic and endothelial cell lineages) efficiently and reproducibly using a serum-free culture system, and have been able to do so with high purity (>95%) and on a

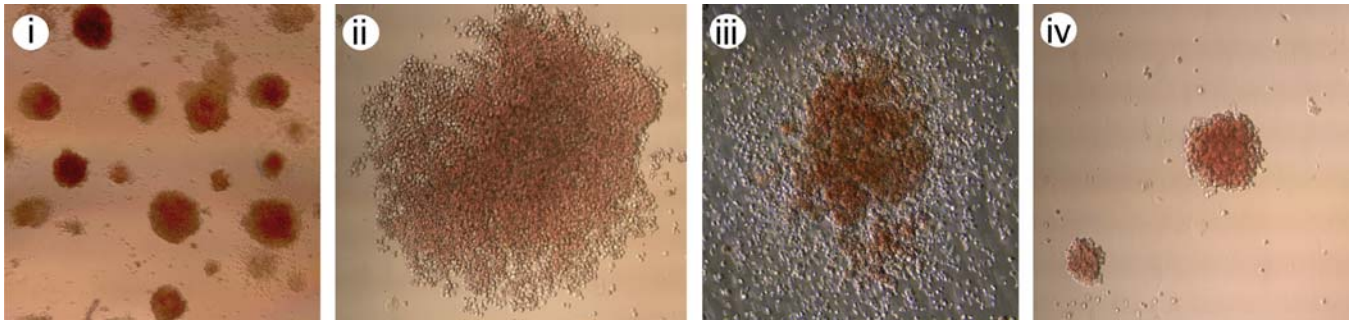

FIGURE 52.1 **Erythroid differentiation potential of human embryonic stem cell–derived hemangioblasts.** These cells showed tremendous colony-forming unit (CFU) capability when cultured in Cephalothin–Fucidin–Cetrimide medium. (i, ii): early burst-forming unit–erythroid (×40, ×100), (iii): large CFU- granulocyte, erythroid, macrophage, megakaryocyte (×200); and (iv): late CFU- erythroid (×100).

relatively large scale [45,46]. A characteristic of the primitive hemangioblast cell is its highly efficient generation of large BFU-E (Fig. 52.1-i and 1-ii), CFU-GEMM (Fig. 52.1-iii), and CFU-E (Fig. 52.1-iv) colonies when cultured in a methylcellulose-based medium. This prompted us to investigate whether hemangioblasts could be used as an intermediate cell source to generate large, clinically relevant quantities of blood components such as erythrocytes and platelets.

Using the hemangioblast system, we generated functional RBCs (blood types A, B, O, and both Rh^+ and Rh^-) on a large scale from multiple hESC lines [33]. Three critical elements allowed us to do this: (1) the efficient generation of hemangioblasts without disruption of their colony-forming environment; (2) expansion of hemangioblasts to erythroblasts in a high cell density (Fig. 52.2i and 2ii); and (3) culture in semisolid methylcellulose-based media to provide optimal conditions for maximum expansion and high erythroid purity (Fig. 52.2i). We generated approximately 10^{10}

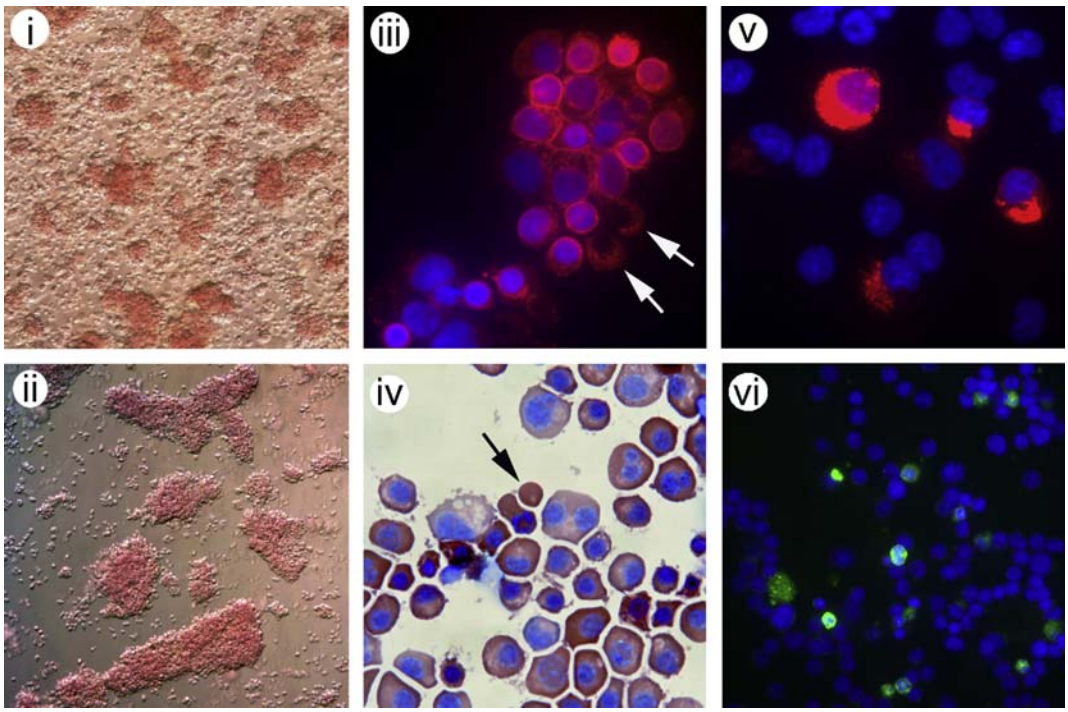

FIGURE 52.2 **In vitro characterization of human embryonic stem cell (hESC)-derived erythroid cells.** (i, ii): Morphology of typical high-density expansion/differentiation of immature hESC-derived erythrocytes cultured with medium containing methylcellulose (×10); (iii): Maturing hESC-derived erythrocytes stain positive for erythrocyte marker CD235a, with a small fraction of cell showing enucleation (*arrows*); (iv): Giemsa–benzidine double staining of maturing hESC-derived erythrocytes showing a high content of intracellular hemoglobin (*brown*). *Arrow* indicates fully enucleated red blood cells (×100); (v): Maturing hESC-derived erythrocytes express A-type surface antigen (*red*, ×100 magnification); (vi): Some hESC-derived erythrocytes express β-chain hemoglobin (*green*, ×100) after elongated culture in vitro. Cellular nuclei in all fluorescence images were stained with DAPI (*blue*).

to 10^{11} erythroid cells per six-well plate of hESCs [33], which is over a thousand-fold more efficient than previously reported methods [38]. Oxygen equilibrium curves of erythroid cells from days 19–21 of differentiation were comparable to normal transfusible RBCs and responded to changes in pH and 2,3-diphosphoglycerate. During the course of our studies, we found that extended in vitro culture facilitated further maturation of these erythroid cells, inducing a progressive decrease in size, increased expression of the erythrocyte cell surface marker, glycophorin A (CD235a), as well as chromatin and nuclear condensation. When the extended culture was performed on OP9 stromal cells, it resulted in the extrusion of the pyknotic nucleus in up to 65% of cells and the generation of enucleated erythrocytes with a diameter of approximately 6–8 μm (Fig. 52.3). At this stage, the erythrocyte population was nearly 100% positive for glycophorin A (Fig. 52.2iii), it had a high content of hemoglobin (Fig. 52.2iv), and it expresses ABO antigen (Fig. 52.2v). Although the cells were found to express fetal and embryonic globin chains, globin chain–specific polymerase chain reaction and immunofluorescent analyses showed that after extended culture, expression of adult β-globin increased from 0% to 15% (Fig. 52.2vi). Overall, these results showed that it is feasible to differentiate and mature hESCs into functional oxygen-carrying erythrocytes on a large scale. The identification of an hESC line with an (O)Rh^- genotype would permit the production of ABO and Rh-compatible (and pathogen-free) "universal donor" RBCs. Considerable effort is still needed to bring hESC-derived RBCs to clinical trials, but these efforts certainly provide a promising lead.

Red Blood Cells Generated From Human Induced Pluripotent Stem Cells

The successful reprogramming of somatic cells into a pluripotent state has been achieved by ectopic expression of various combinations of transcription factors [11,13]. The derivation of these iPSCs is less controversial than that of hESCs; thus, they open up an exciting new route to obtain PSCs. Moreover, that iPSCs can be produced in a patient-specific manner will eliminate the issue of immunorejection in cell, tissue, or organ replacement therapies in the future. A number of groups including our own have published methods for generating RBCs from iPSCs

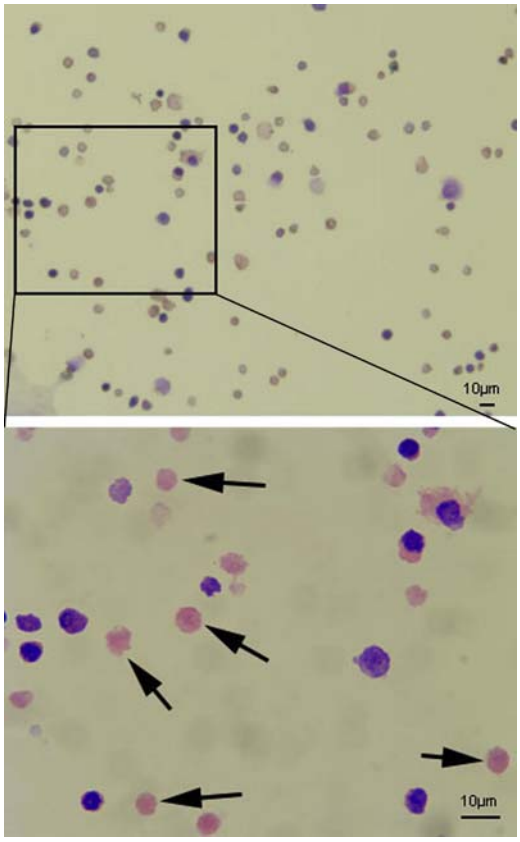

FIGURE 52.3 **Enucleation of erythrocytes derived from human embryonic stem cells in vitro.** (*Top*): Low-magnification image of Giemsa-stained enucleated erythrocytes (×20); (*bottom*): High-magnification (×100) image of enucleated erythrocytes (*black arrows*).

[47–59]. These erythrocytes possess classic morphology, express glycophorin A, and have abundant hemoglobin content. As observed in the hESC differentiation systems, regardless of their origin, iPSC-generated RBCs express mostly fetal hemoglobin (hemoglobin F). RBCs expressing hemoglobin F are slightly less efficient than those expressing adult hemoglobin for oxygen delivery. However, patients who retain expression of hemoglobin F in adult life are not anemic. Thus, the fetal phenotype of RBCs generated from iPSCs that are derived from patients with hemoglobinopathies is not necessarily a barrier for autologous transfusion. Kobari et al [52] and Yang et al. [57] reported the switch from fetal to adult hemoglobin after infusion of iPSC-derived nucleated erythroid precursors into mice, which indicates that these cells possess the potential to turn on the expression of the globin gene under appropriate in vivo conditions. Giani et al. [60] demonstrated that targeted suppression of SH2B3 in primary human hematopoietic stem and progenitor cells enhanced the maturation and overall yield of in vitro–derived RBCs. Olivier et al. [61] described a GMP-compatible, feeder-free, and serum-free method to produce large numbers of erythroid cells from human PSCs. This multistep protocol combines cytokines and small molecules to mimic and surpass the early stages of development and produces more than 99% of CD235a-positive cells. Moreover, their protocol supports extensive expansion, with a single human PSC producing 50,000–200,000 erythroid cells. The erythroid cells produced exhibit a definitive fetal hematopoietic type, with 90–95% fetal globin and a variable proportion of embryonic and adult globin proteins.

Although these studies provided proof-of-principle, our study revealed some intrinsic molecular and cellular abnormalities in the iPSC derivatives, such as increased apoptosis, limited CFU capability, and limited expansion [51]. The exact cause(s) of these abnormalities is unclear but may result from alterations caused by the modified genome of virally reprogrammed cells. Despite transgene silencing after reprogramming, Dias et al. [50] reported a background of transgene expression in differentiated cells obtained from transgenic iPSCs and showed that it can affect their properties. However, Dias et al. [50] observed no difference in erythroid differentiation between transgene-free and transgenic hiPSC lines. Our study [30] and unpublished data showed that viral-free, especially iPSCs generated with messenger RNA (mRNA) transfection, have much improved hematopoietic differentiation and expansion capability compared with human iPSCs carrying viral DNA. Both large-scale RBCs and megakaryocytes and platelets were produced with mRNA-generated iPSCs, which is comparable to hESC-derived counterparts. More research will be needed to figure out the mechanism underlying the disparate observations.

Red Blood Cell Generation by Direct Conversion of Somatic Cells

Another new approach to RBC generation involves the direct dedifferentiation or conversion of human somatic cells to multipotent hematopoietic progenitors, thus bypassing the need for PSC-like hESCs or iPSCs altogether [32,62]. Forced ectopic expression of OCT4 directly transdifferentiates fibroblasts into $CD45^{+}$ hematopoietic progenitors, and after exposure to EPO, these cells were able to produce erythroid lineage cells that expressed high levels of adult β-globin and low levels of fetal γ-globin and were capable of enucleation [62]. Similarly, Liu et al. [32] reported a method to generate erythrocytes from human hair follicle mesenchymal stem cells by enforcing OCT4 gene expression and cytokine stimulation. Erythroid cells generated from human hair follicle mesenchymal stem cells expressed mainly the adult globin chain with minimum levels of the fetal globin chain. Furthermore, these cells also underwent multiple maturation events and formed enucleated erythrocytes with a biconcave disc shape and the morphology of mature and functional RBCs. The limitation of this approach is the high level of OCT4 expression in somatic cells, which can be achieved only by viral infection. Viral integration in the genome limited the usefulness of this approach.

Where Do We Go From Here?

The manufacture of safe and effective RBC substitutes will alleviate many of the risks, complications, and hardships associated with donor-dependent RBC sources. Significant progress has been made by manipulating the differentiation potential of hESCs and iPSCs and driving them toward erythrocyte development. In vitro differentiation systems that can be scaled up for the mass production of RBC substitutes have been developed. The hemangioblast methodology described in this chapter represents one such possibility. Despite many exciting advances with in vitro culture systems, problems associated with the final stages of erythrocyte maturation, namely enucleation and globin switching, will still need to be fully resolved before hESC- and iPSC-derived RBCs can be produced in a stroma-free manner, scaled up for mass production, and brought to the clinic. Direct conversion of somatic cells into RBCs represents an alternative to solving the globin-switching dogma, but the low efficiency

and use of viral vector limits its clinical application. Another obstacle to applying all of these methods is that they are expensive to produce the sufficient number of RBCs that are applicable in the clinic.

MEGAKARYOCYTES AND PLATELETS

Megakaryopoiesis

Because of their role in clot formation and blood vessel repair, platelets are essential for the cessation of bleeding; to maintain an abundant supply of them is vitally important. MK, the large multinucleate precursors to platelets, provide a constant, renewable source of platelets to the blood system and are themselves produced through a process called megakaryopoiesis. As previously mentioned, HSCs differentiate into CMPs, which then undergo significant proliferation and expansion as they further mature and differentiate. Once exclusively committed to the MK lineage, however, they retain only limited expansion capacity. On a cellular level, MK maturation involves many changes such as an increase in expression of the cell surface markers GPIIb/IIIa (also known as CD41 or αIIb/βIII integrin receptor) and GPIb/GPIX/GPV receptors and an increase in cytoplasmic and nuclear mass, with cells expanding to 50−100 μm in diameter, resulting in nuclear polyploidization and cells up to 128 N [63,64]. On a molecular level, thrombopoietin (TPO) is the primary physiological regulator of MK and platelet generation [7]. However, numerous other cytokines, growth factors, and small molecules have been found to be important as well. Multiple cytokines have been reported to stimulate the proliferation of MK progenitors synergistically [65,66], whereas transforming growth factor-β1 and platelet factor 4 have been found to inhibit their development and maturation [67]. Downstream of cytokine signaling pathways, various transcription factors orchestrate the progressive lineage commitment and maturation of developing MKs [68−72].

Biogenesis of Platelets

Although not mutually exclusive, two models have been proposed to describe the assembly and release of platelets from MKs into the bloodstream, a process known as thrombopoiesis [6,73]. In the global fragmentation model, cellular processes termed proplatelets undergo mutual detachment to produce small functional platelets within the MK cytoplasm, which then explodes and releases the cell's entire content of platelets simultaneously. This theory, conceived in the 1970s, is supported by electron microscopy of mature MKs, as well as in vivo and ex vivo observations of platelet release. More recent experimental evidence lends support to the proplatelet model of platelet biogenesis. In this model, proplatelets actually extend from the mature MK cell body, traverse the vascular endothelium, and enter the BM sinusoids, where the shear force of the bloodstream facilitates release of nascent platelets from proplatelet ends. On a molecular level, thrombopoiesis is thought to be a highly coordinated process, with sophisticated reorganization of membrane and microtubules and precise distributions of granules and organelles [74]. It also appears as though localized apoptosis may have important roles in proplatelet formation and platelet release [75]. Despite these advances in our understanding of platelet biogenesis, mechanistic details remain to be elucidated.

Generation of Megakaryocytes and Platelets From Adult Stem Cells and Somatic Cells

Various sources of adult HSCs have been used to study MK and platelet differentiation. Choi and colleagues were the first to report that functional platelets could be produced from $CD34^+$ cells isolated from PB [76]. During 10−12 days of culture, aplastic canine serum was used to promote in vitro MK lineage commitment and maturation and was then replaced by human serum to enhance platelet production from the MKs. Platelets generated from this system demonstrated aggregation capacity when stimulated with either adenosine diphosphate or thrombin, the physiological agonists for normal blood platelets. Subsequent studies showed that HSCs from PB, BM, and CB are also all capable of producing MKs and functional platelets [77−79]. Several groups reported the direct conversion or dedifferentiation of somatic cells into MKs and platelets [79a,80,80a], although the in vivo function of these platelets was not examined. Another strategy is to generate an immortalized MK progenitor cell lines from hematopoietic progenitors with virally transduced genes [81], but the methods involving viral transduction may not be considered appropriate for clinical-grade manufacturing. Yet, the limited in vitro expansion capabilities of primary $CD34^+$ and somatic cells make these cells unable to replace donation as a source of platelets. For this reason, human PSCs may be a better starting cell population for large-scale in vitro production.

Megakaryocytes and Platelets Generated From Human Pluripotent Stem Cells

After initial success with murine ES cells [82–84], MK differentiation from human PSCs including both hESCs and iPSCs was achieved in coculture systems with animal stromal cells [31,35,36]. In these studies, Takayama et al. [35,36] cocultured hESCs or iPSCs on C3H10T1/2 stromal cells for 14–15 days and then handpicked sac-like structures containing hematopoietic progenitors and replated single cell suspensions onto fresh stroma in medium containing TPO, SCF, and heparin for 9–23 days. During this time, polyploid, CD41a/CD42b double-positive MKs emerged and produced platelets with characteristic morphology, as assessed by electron micrography. A variety of in vitro tests confirmed PSC-platelet functionality. In another study by the researchers, a laser-induced vascular injury model was used to show that their iPSC-derived platelets readily incorporate into newly developing thrombi in vivo [36].

The use of both serum and animal feeder layers throughout these studies hinders the ability of these methods to be adapted for clinical use. In addition, because handpicking of PSC sacs is both time- and labor-intensive, alternative methods will likely have to be developed for large-scale production. Pick et al. [85] and Liu et al. [32] reported feeder-free and xeno-free systems to generate MKs from PSCs. Several crucial reagents were also replaced with US Food and Drug Administration–approved pharmacological reagents. However, the generation of functional platelets was not reported in these two studies. We were able to use the serum- and feeder-free hemangioblast system, as described earlier for RBCs, to generate MKs efficiently [34]. Yet, similar to the studies by Takayama et al., we found that efficient platelet generation from hemangioblast-derived MKs still requires a conventional stroma coculture. To this end, we developed a well-defined and completely serum- and feeder-free system to differentiate human PSCs into MKs and functional platelets in less than 20 days, which greatly increases its ability to be developed for clinical use [30]. Using this method, large quantities of cryopreservable MK progenitors were produced from human PSCs, which allowed billions of platelets to be produced rapidly upon thawing of these cells. The improved scale of in vitro–generated PSC platelets provided enough materials to perform light transmission aggregometry (LTA), which is the most common method used in clinical and research laboratories to assess platelet function [86]. LTA has not been performed with platelets generated from human PSCs in vitro, presumably owing to its requirement for a large quantity of fresh platelets. As shown in Fig. 52.4C, human PSC platelets responded to thrombin stimulation in forming aggregates. However, the process was slow and aggregation was weak compared with PB platelets, but stronger and faster than human CB platelets, which is consistent with previous observations [87–89]. These results are a significant improvement over in vitro $CD34^+$ cell-derived platelets, which showed no aggregation by LTA [90]. Differential-interference contrast and electron microscopy analyses showed that ultrastructural and morphological features of PSC-derived platelets were indistinguishable from those of normal blood platelets (Fig. 52.4A). Human PSC platelets form aggregates, lamellipodia, and filopodia after activation and circulate in macrophage-depleted animals and incorporate into developing mouse thrombi in a manner identical to human platelets (Fig. 52.4B; [30]. By knocking out the β2-microglobulin gene, we generated platelets that are negative for the major histocompatibility antigens (Fig. 52.5). The scalable generation of HLA–ABC-negative platelets from a renewable cell source represents an important step toward generating "universal" platelets for transfusion as well as a potential strategy for managing platelet refractoriness.

Improving the Efficiency for In Vitro Platelet Production

These studies provide an important proof of principle for the in vitro manufacturing of functional platelets from different cell sources. However, the efficiency of platelet production will need to be significantly improved to achieve clinically relevant yields [91]. Reems et al. [92] used an in vitro culture system to demonstrate the feasibility of producing functional platelets for clinical use. Using this protocol, the calculated yield of platelets from 5×10^6 $CD34^+$ cells is $1.26–1.68 \times 10^{11}$, which is equivalent to approximately 3 units of donor-derived platelets. However, these calculations are based on extrapolating data from experiments using just 500 starting $CD34^+$ HSCs. A three-dimensional culture system described by Sullenbarger et al. [93] provides additional evidence that it is possible to produce platelets in scalable quantities. In this system, about 20 platelet-like particles were produced from each input $CD34^+$ cell. Thon et al. [94] developed a scalable microfluidic platelet bioreactor that reproduces the BM microenvironment ex vivo, synchronizes proplatelet generation, and strikingly increases the yields of functional platelets by a magnitude of two compared with static conditions from different sources of MKs; this represents the critical next step in creating an alternative source of functional human platelets for infusion. Although this progress is significant, the efficiency is still low compared with greater than 2000 platelets/MK in BM, which remains the biggest challenge to reaching a clinically relevant scale of ex vivo platelets. Considering the optimal in vivo capacity

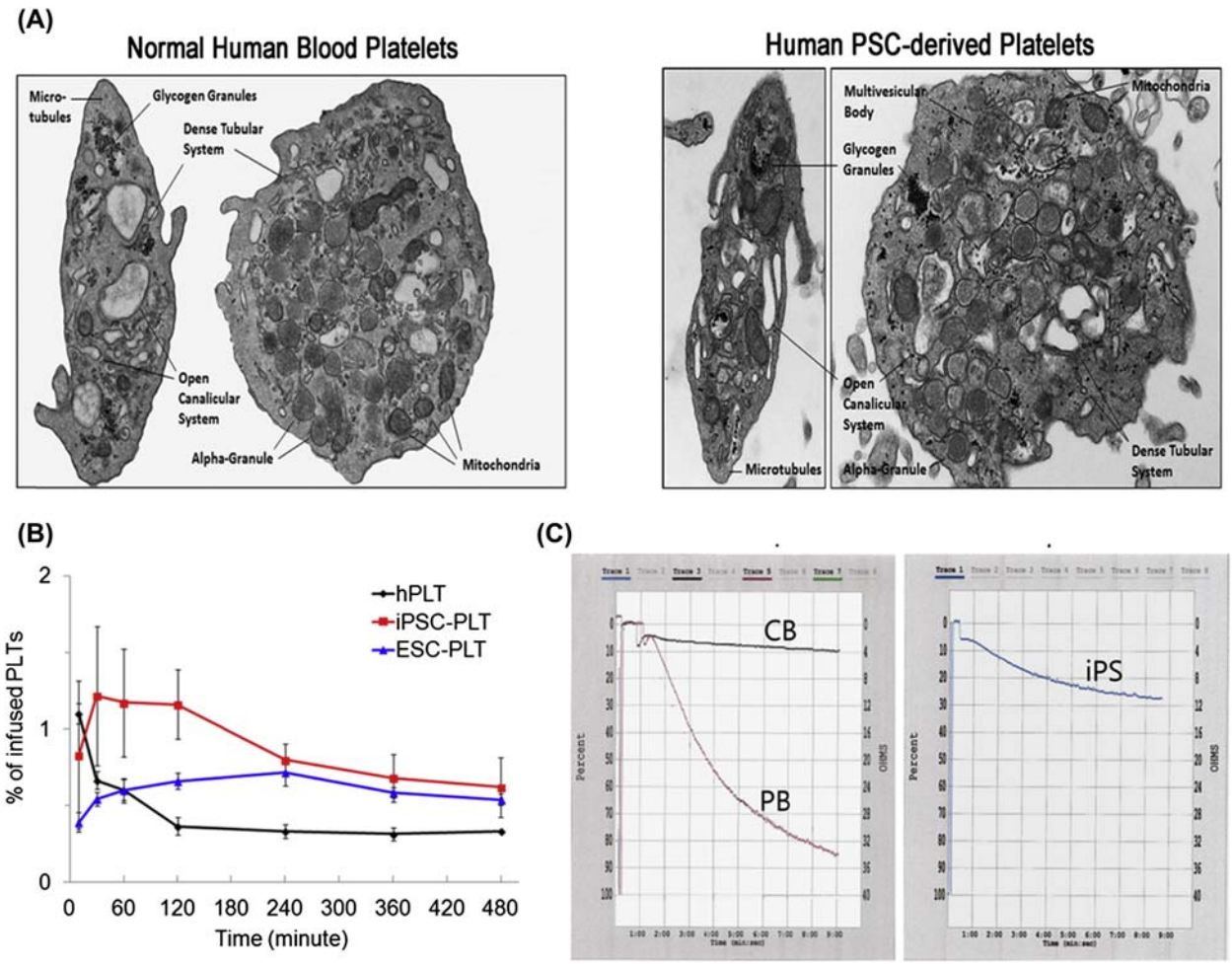

FIGURE 52.4 **Characterization of induced pluripotent stem cell (iPSC)-derived platelets (PLT) in vitro and in vivo.** (A) Thin-section electron micrographs of (*left*) human blood and (*right*) iPSC platelets. (B) Comparative kinetics of human blood platelets (hPLT), human embryonic stem cell platelets, and iPSC platelets in immune-deficient mouse blood system (mean ± SD, n = 5). (C) Aggregation assay of platelets from human peripheral blood (PB), umbilical cord blood (CB), and iPSC platelets stimulated with 1 U of thrombin using Chronolog aggregometer. *PSC*, pluripotent stem cell. *This research was originally published in Stem Cell Reports: Feng, Q. et al. (2014). Scalable generation of universal platelets from human induced pluripotent stem cells. Stem Cell Reports 3, 1–15.*

of MK development from HSCs and platelet production from MKs, both the initial hematopoietic amplification stage and downstream platelet biogenesis could stand to be optimized. Strategies to increase the efficiency of MK and platelet production include the development of novel culture systems that mimic the in vivo BM microenvironment as well as the optimization of media formulations including concentrations of cytokines, small molecule mimetics, and nutrients [91,95]. In addition, physiological parameters such as pH, media viscosity, and oxygen levels all may be optimized for increased platelet biogenesis. Finally, in vivo observations that helped to formulate the proplatelet model of platelet biogenesis suggest that shear force could have an important role in platelet release [6,74]. Adaptation of such a mechanical force in culture systems may also significantly promote proplatelet growth and platelet release, as demonstrated by Thon et al. [94].

HEMATOPOIETIC STEM CELLS

Hematopoietic cancers, including leukemia, Hodgkin and non-Hodgkin lymphoma, myeloma, and myelodysplastic syndromes, result from the unregulated proliferation of hematopoietic cells. A promising treatment for hematopoietic malignancies is the transplantation of HSCs collected from PB, BM, or CB of HLA-matched donors into patients after radiation or chemotherapy. Allogeneic HSC transplantation is the only treatment that can cure

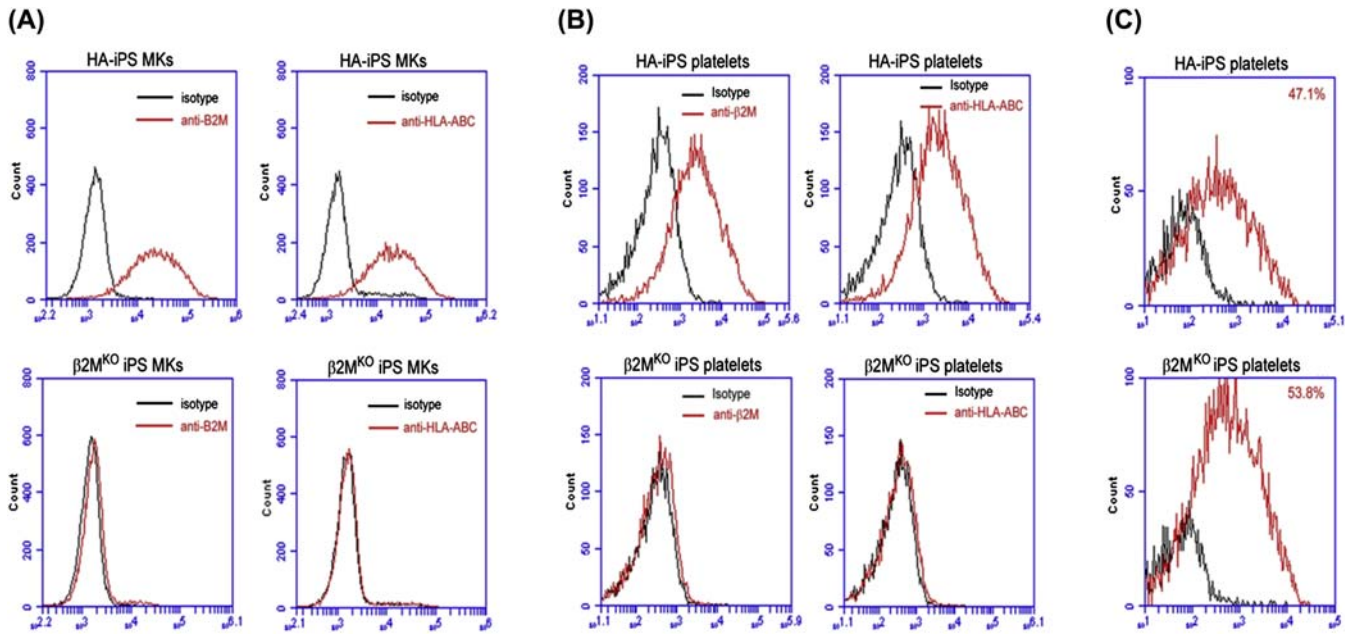

FIGURE 52.5 **Generation and characterization of universal platelets from human induced pluripotent stem cell (iPSCs).** (A) human leukocyte antigen (HLA)-ABC and β2-microglobulin (β2M) expression in wild-type hemophilia A (HA)-iPSCs and $\beta2M^{KO}$ iPSC-derived megakaryocytes (MKs); (B) HLA-ABC and β2M expression in wild-type HA-iPSCs and $\beta2M^{KO}$ iPSC-derived platelets; (C) Platelet activation measured by PAC-1 binding with (*red histogram*) or without thrombin (*black histogram*) in wild-type HA-iPSC platelets and $\beta2M^{KO}$ iPSC platelets. *This research was originally published in Stem Cell Reports: Feng, Q. et al. (2014). Scalable generation of universal platelets from human induced pluripotent stem cells. Stem Cell Reports 3, 1–15.*

chronic myelogenous leukemia. However, HSCs from HLA-matched donors are often scarce, which has been a bottleneck for treating blood cancers, especially for patients from minor ethnic groups.

Several strategies have been developed to generate HSCs from human PSCs [29,96], which possess the potential to serve as an inexhaustible and donorless source of HSCs for transplantation therapy. In addition, iPSCs have the potential for curative treatment for a variety of genetic diseases that affect PB and BM-derived cells. Hematopoietic cells derived from PSCs have been successfully transplanted into immune-deficient NOD-SCID mice, which showed short-term as well as long-term BM engraftment capabilities [97–102]. Similarly, Amabile et al. [103] and Suzuki et al. [104] independently demonstrated that $CD34^+$ $CD45^+$ cells isolated from teratomas formed by human PSCs in immune-deficient mice were able to engraft and reconstitute hematopoietic cells in serial transplantation experiments. These studies suggest that functional HSCs can be derived from human PSCs. However, in all of these studies, BM repopulation was invariably low, only rarely exceeding 1–2%.

The causes for the observed consistently poor engraftment of PSC-derived hematopoietic cells in immune-deficient mice may be multifactorial. First, the PSC-derived hematopoietic cells themselves may be defective, losing some properties that are critical for engraftment, such as the potential of differentiating into endothelial cells. It has been demonstrated by several groups that functional HSCs derived from CB and BM exhibit hemangioblastic activity, producing both hematopoietic and vascular cells in response to vascular injury [105–107]. Hooper et al. [108] found that irradiation caused severe regression of BM sinusoidal endothelial cells, whereas HSC transplantation into these myeloablated mice induced rapid regeneration of the regressed sinusoidal endothelial cells with robust HSC engraftment. However, regeneration inhibition of the damaged sinusoidal endothelial cells prevented engraftment and replenishment of HSCs, leading to hematopoietic failure in these animals.

Second, PSC-derived hematopoietic progenitor cells are of embryonic origin and might have decreased potential for BM engraftment in the marrow microenvironment of adult animals. Experimental evidence supportive of this possibility was presented by Yoder et al. [109]. These investigators observed that mouse yolk sac hematopoietic cells failed to engraft when transplanted into congenic adult recipients but produced durable engraftment and multilineage reconstitution of peripheral blood cells when infused into myeloablated newborn pups. This suggests that the cellular microenvironment has a critical role in proliferating and differentiating hematopoietic progenitor cells.

In effort to overcome challenges associated with HSC generation from human PSCs, several groups took the strategy of directly reprograming adult somatic cells into hematopoietic cells. Riddell et al. [110] demonstrated

that transient expression of six transcription factors (Run1t1, Hlf, Lmo2, Prdm5, Pbx1, and Zfp37) converted mouse committed hematopoietic progenitors into induced-HSCs that possess multilineage differentiation potential, reconstitute stem and progenitor compartments, and are serially transplantable. Similarly, Sandler et al. [111] showed that enforced expression of transcription factors FosB, Gfi1, Runx1, and Spi1 directly converted human endothelial cells into cells consisting of HSCs with long-term engraftment potential. Cheng et al. [112] reported that hematopoietic transcription factors Scl, Lmo2, Runx1, and Bmi1 converted mouse fibroblasts into "induced hematopoietic progenitors" that engrafted immune-deficient mice, generating myeloerythroid and B-lymphoid cells for up to 4 months in vivo. These studies suggest that enforced expression of a small number of transcription factors can reprogram adult somatic cells into engraftable HSCs, but the use of viral or DNA-based vectors confers another threat to clinical application.

PERSPECTIVES

Limitations in the supply of RBCs and platelets can have potentially life-threatening consequences for transfusion-dependent patients with unusual or rare blood types, particularly those who are alloimmunized. Although CB, BM, and PB have been investigated as sources of progenitors for the generation of large-scale transfusable RBCs and platelets [24–26,92,93], it is clear that even after expansion and differentiation, these progenitors represent donor-limited sources of RBCs and platelets. Human PSCs, especially virus-free human iPSCs [113–115], represent a new source of stem cells that are donorless source of RBCs and platelets for human therapy. Advances in gene editing technology allow the generation of PSC cells that can be used to produce (O)Rh-negative "universal" RBCs and O-type HLA major–negative "universal" platelets, which could eliminate tissue incompatibility issues altogether [116]. As for HSCs, de novo generation from human PSCs remains the most challenging obstacle. Further studies will be needed to understand the basic mechanism of HSC development in vivo and to apply this knowledge to develop a system that mimics the events accurately in vitro. The success of generating engraftable HSCs from human PSCs will be a "Holy Grail" for HSC fundamental biology and hematopoietic malignancy therapy.

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S.-J. Lu and R. Lanza are employees of the Vcanbio Center for Translational Biotechnology and the Astellas Institute for Regenerative Medicine, respectively, companies in the field of regenerative medicine and cell therapy.

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CHAPTER

53

Cartilage Tissue Engineering

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CARTILAGE AND CARTILAGE REPAIR

Cartilage is a connective tissue that functions to provide form, strength, and support. Three types of cartilage are distinguished by their molecular components in the extracellular matrix (ECM), their anatomical location, and their function. Hyaline (articular) cartilage has a white glassy appearance and is found primarily in articulating joints. Its ECM is mainly composed of water, hyaluronate, proteoglycans, and type II collagen. Hyaline cartilage functions to provide stable movement with minimal friction. It has high viscoelasticity and demonstrates an excellent ability to provide resistance to compression and cushion the impact caused by physical load during movement [1]. Elastic cartilage is distinguished by the presence of elastin in the ECM. Elastic cartilage provides a structural function; it is represented by the support it provides in the external ear. Finally, fibrocartilage has a higher proportion of type I collagen in its ECM. Fibrocartilage is found in the meniscus and intervertebral disc and at the distal region of tendons and ligaments in apposition to bone. It provides tensile strength and counters compression and shear forces [2].

All three types of cartilage feature a sparse cellularity, limited blood supply, and lack of neural innervations. These cartilages have intrinsically poor reparative capabilities owing to their purported inability to form a clot to attract the necessary fibroblasts and start subsequent tissue synthesis for repair [3]. Once defects, even small ones, are initiated in cartilage, the degradation process is progressive [4]. One irreversible consequence of the destruction of articular cartilage is arthritis, a leading cause of disability. Osteoarthritis (OA), the most common type of arthritis, is characterized by articular cartilage loss and degeneration, subchondral bone thickening, osteophyte formation, and joint inflammation [5]. OA is widespread globally in 60–70% of people aged older than 65 years [6–8]. Over 21 million people have this disease in the United States and 10% of cases are estimated to be caused by previous trauma to weight-bearing joints, which is classified as posttraumatic OA (PTOA) [9]. PTOA develops not only in elderly people, but also in young people experiencing the results of previous trauma. Once a cartilage defect is present, cartilage matrix continues to be lost while the surrounding tissues become more inflamed and contribute to further cartilage matrix destruction [10]. This causes significant pain, disability, and morbidities, strongly affecting an individual's capacity to live a full and active life.

Surgical treatment options available for focal cartilage repair include microfracture and osteochondral autografting. Microfracture may be considered a current standard for cartilage repair and is a low-cost and minimally invasive procedure [11,12]. This technique employs subchondral drilling to initiate cartilage repair by inducing bleeding, enabling mesenchymal progenitor cells from the bone marrow to migrate into the lesion site. After this procedure, the repair tissue appears to be a cartilage-like substitute but is primarily composed of fibrocartilage. Unfortunately, the repair fibrocartilage has inferior quality and longevity compared with the native hyaline cartilage, thus delaying cartilage degeneration for only a few years [12]. Osteochondral autografting or mosaicplasty is a technique of autotransplantation in which osteochondral plugs are harvested from non–weight bearing or low–weight bearing regions of the joint and implanted into defects that have been prepared and sized. In clinical testing, survival of the transplanted hyaline cartilage has been reported in 85% of patients, with a 91% good to excellent clinical outcome reported by patients observed for 3–6 years [13,14]. In addition, follow-up 10 years after surgery demonstrated improved clinical outcomes compared with microfracture [15]. However, the cartilage autografts have many problems including limited donor tissue availability, donor site injury, scarring, and pain, prompting

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the development of an innovative bioengineered therapy called autologous chondrocyte implantation (ACI), which will be discussed later in this chapter [16,17]. Allograft tissue transplants address the donor tissue availability challenge and are a fast-growing therapy.

TISSUE ENGINEERING FOR CARTILAGE REPAIR

To overcome the treatment obstacles of available surgical options for cartilage repair, the reconstruction of cartilage using tissue engineering techniques has attracted tremendous attention. Tissue engineering is a multidisciplinary field that applies the principles of engineering, life sciences, and cell and molecular biology to the development of biological substitutes that restore, maintain, and improve tissue function [18]. The historical approach to cartilage engineering is to select and optimize the following components to be used individually or in combination to regenerate organs or tissues [1]: reparative cells that can form a functional matrix [2], an appropriate scaffold for transplantation and support, and [3] bioreactive molecules such as cytokines and growth factors that will support and choreograph formation of the desired tissue [19]. Biological cues and important biological factors that promote tissue repair in the local in vivo tissue environment continue to be discovered and developed, propelling the field forward. For example, the innate and adaptive immune systems are newly appreciated factors for successful tissue engineering. The immune system participates in many facets of tissue repair via scavenging dead cells and debris, inducing vascularization of injured tissue, and recruiting progenitor cells to tissue [20–22]. More specifically, Badylak discovered that macrophage phenotype is important in muscle remodeling, and that macrophage phenotype is influenced by ECM scaffolds implanted into a muscle wound [23]. Sadtler found that $CD4^+$ T-helper 2 cells were necessary for proregenerative macrophage polarization and subsequent muscle regeneration after treatment with ECM scaffolds, and concluded that the adaptive immune system as well as the innate immune system are important for functional tissue regeneration with a scaffold [24]. Although little is known about how either innate or adaptive immune cells may be involved in cartilage regeneration, it is likely to be a future focus for the field (Fig. 53.1).

Cartilage Surface Modification

Engineering therapies aimed to prevent cartilage deterioration after injury are valuable considering that cartilage is extremely difficult to repair. Because lubrication of the cartilage surface is extremely important for its proper

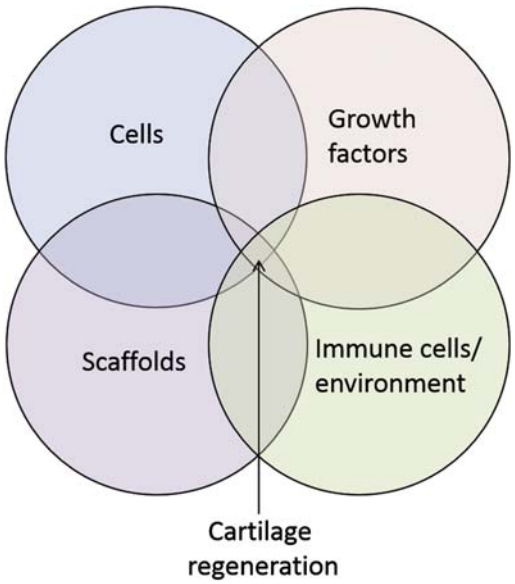


FIGURE 53.1 Revised approach to tissue engineering triad. Cells, growth factors, scaffolds, and immune cells and the immune environment contribute to engineered cartilage regeneration. The immune system contributes inflammatory and antiinflammatory signals, as well as antigen-specific and nonspecific recognition of the engineered scaffold, which may dictate engineered tissue success.

movement without friction and mechanical degradation, viscosupplementation is a common procedure. Viscosupplementation replenishes the molecules in the synovial fluid that naturally lubricate the cartilage surface, most commonly hyaluronic acid. One strategy is to enhance viscosupplementation with synthetic molecules such as a hyaluronic acid (HA) binding peptide to enhance the retention of HA further at the joint surface [25,26]. Synovial fluid can also be enhanced with synthetic charged polymers and synthetics with large molecular brush structures [27]. Synthetic lubricin, synthetic mimics of lubricin, and recombinant lubricin have also been created to replenish lubricin lost at the beginning of cartilage degeneration before major structural cartilage changes have occurred [28]. These lubricin mimics bind HA to the cartilage surface, increasing boundary layer lubrication. Synthetic scaffolds for directing cartilage tissue repair will be covered in the scaffold section.

Cell Types for Cartilage Repair

Different cell sources are available to provide reparative tissue including differentiated cells, mesenchymal stem cells (MSCs), and embryonic progenitor cells. Chondrocytes and MSCs are the two most investigated cell sources for cartilage tissue engineering. Chondrocytes can be isolated from tissue biopsies and cultured ex vivo to achieve the required cell number for engineering new tissue. Chondrocytes have been expanded ex vivo for clinical applications as a US Food and Drug Administration (FDA)-approved therapy [17]. However, a major limitation of chondrocytes is their need to be expanded because of the small amount of cartilage that can be biopsied and the tendency of the chondrocytes to dedifferentiate rapidly in monolayer culture during expansion [29,30]. Tissue culture material and scaffold type can influence chondrocyte phenotype. Culturing cells on hard, flat surfaces results in a loss of the typical chondrocyte rounded morphology, decreased cartilage-specific gene expression, and increased fibrotic gene expression [31]. Three-dimensional (3D) culture and scaffolds preserve the round shape and chondrocyte phenotype to promote chondrogenesis and increased type II collagen and decreased type I collagen synthesis compared with chondrocytes in monolayer culture [32,33].

MSCs represent an alternative to chondrocytes as a cell source for cartilage tissue engineering [34]. MSCs have the advantage of being able to be expanded in vitro in an undifferentiated state while retaining the ability to differentiate after exposure to suitable stimuli [35]. Like chondrocytes, a 3D culture environment for cartilage engineering with MSCs is superior to monolayer culture. Winter et al. compared chondrogenic gene expression and morphology from MSCs derived from bone marrow and adipose tissue [36]. That study demonstrated only partial cell differentiation in monolayer culture; however, bone marrow–derived MSCs showed improved chondrogenesis in 3D culture. In addition to their ability to differentiate into chondrocytes, MSCs have immunomodulatory properties, can migrate to sites of inflammation, and can modulate lymphocyte cell function through several growth factors and cytokines including transforming growth factor-β1 (TGF-β1), nitric oxide, and interleukin-10 (IL-10) [37]. This immunomodulation is especially important when considering the destructive environment in which a cartilage construct is likely to be implanted. MSC therapies for OA are undergoing clinical trials, but not many long-term results are available. Therapies being tested include injection of MSCs isolated from different sources, MSC injection with additional biological factors, and injection of ex vivo expanded MSCs [38]. A clinical trial of bone marrow–derived MSC injections showed improved *Western Ontario* and McMaster Universities Arthritis Index scoring over 1 year after treatment, indicating decreased pain and increased function. In addition, X-ray and magnetic resonance imaging findings indicated that MSC treatment may halt the progression of cartilage loss [39]. However, a common problem with MSC therapy is the poor delivery and retention of MSCs at the cartilage defect site. There are preclinical models aimed at optimizing MSC delivery; however, most clinical trials inject MSCs free of scaffold [38].

Bioscaffolds in Cartilage Repair

Tissue engineering scaffolds are designed to provide a 3D environment to support and direct cellular processes in their migration, proliferation, and differentiation toward functional tissue. Scaffolds can be applied with cells; however, applying scaffold without cells is becoming more attractive because cell therapy is more costly. The selection of bioscaffolds for cartilage engineering requires complex mechanical properties that can support cellular functions, biocompatibility, capability of waste and nutrient transport, and sufficient structural integrity for joint reconstruction. Both natural and synthetic materials have been applied as cartilage tissue engineering scaffolds in a variety of forms, including fibrous structures, porous sponges, woven or nonwoven meshes, and hydrogels.

Natural Scaffolds

Collagen

Collagen is the primary structural protein found in both bone and cartilage [40,41]. As such, collagen-based scaffolds are theoretically capable of supporting chondrocyte attachment and function. They are also biocompatible and biodegradable. Collagen scaffolds have been used in a wide variety of forms such as gels, membranes, and sponges into which cells and/or bioactive factors may be introduced [42,43]. Pieper et al. employed a cross-linked porous type II collagen sponge to support the proliferation and differentiation of chondrocytes under cell culture condition up to 14 days [42]. Yokoyama et al. cultured MSCs in a collagen gel matrix in a chondrogenic medium supplemented with bone morphogenetic protein-2 (BMP-2), (TGF-β3), and dexamethasone [44]. The constructs were characterized by a downregulation of type I collagen and upregulation of type II collagen and the cartilage-related proteoglycans aggrecan, biglycan, and decorin. The maximum size of cartilaginous tissue produced was 7 mm in diameter and 0.5 mm in thickness, still too small for partial-thickness cartilage repair. These cell-based studies indicate some of the disadvantages of collagen scaffolds. Collagen gels allow for uniform mixing of cells and matrix, and for extensive molding and shaping of tissue, but they tend to be fragile until new matrix is laid down. Solid collagen scaffolds such as membranes or sponges exhibit greater initial mechanical strength, but at the cost of less flexibility in shaping and a greater risk of nonuniform cell seeding. Collagen remains a useful scaffold with which to study 3D cell culture, but these disadvantages noted weigh against its use in clinical applications.

Hyaluronic Acid

HA is a polysaccharide that is naturally found in the ECM of articular cartilage and in synovial fluid; it is responsible for the high lubricity of the cartilage surface. It is composed of alternating residues of *N*-acetyl-D-glucosamine and D-glucuronic acid. As with collagen, interest focused on HA as a potential scaffold for cartilage engineering results from its intimate association with chondrocytes in vivo. Intraarticular HA injection has been used to treat symptoms of OA with large world markets and sales and enhances cartilage lubrication and exerts many biologic effects on cells. HA has been shown to have a stimulatory effect on chondrocyte production of type II collagen and proteoglycan [45]. HA also has many immunomodulatory properties. HA less than 50 kDa is considered to be low–molecular weight (LMW), inflammatory HA; at 10^3–10^4 kDa and greater, it is antiinflammatory [46]. HA interacts with many cell receptors including receptor of hyaluronan-mediated motility, CD44, and the immune-related receptors Toll-like *receptors* 2 and 4, giving it the ability to affect inflammation and cell migration, both of which are important aspects of wound healing [47]. It is also thought that LWM HA may provide a danger signal to immune cells to attract them to the site of tissue degeneration to aid in tissue debris clearance, which can damage tissue when occurring chronically [48]. These properties make HA a highly dynamic and important molecule to include in scaffold designs, especially considering that HA makes up a significant part of the chondrocyte pericellular matrix [49] Clinical trials using HA to treat OA have found variable results in the efficacy of HA to reduce OA progression. More research on how variation in different HA products such as molecular weight and cross-linking affects clinical outcome is needed to optimize HA therapeutic effect [50].

Alginates

Alginates are polysaccharides derived from seaweed. They are composed of a family of linear mannuronate/guluronate copolymers that differ in their specific sequences and overall compositions [51]. When exposed to a divalent cation (usually calcium, for the sake of biocompatibility), the linear alginate polymers ionically cross-link to form a porous hydrogel. This allows the uniform seeding of chondrocytes and bioactive factors within the alginate hydrogel, as well as their release, if desired, by exposure to a cation chelating agent such as ethylenediamine tetraacetic acid. Alginates may also be covalently modified to enhance properties such as cell adhesion [51–53] or to fix bioactive factors in place [54–56].The clinical translation of alginates as in vivo scaffold for cartilage repair may be limited by the potential calcification of the constructs [56]. On the other hand, compared with the monolayer cell culture of chondrocytes, alginate matrices provide a convenient means to preserve or reestablish the characteristic chondrocyte phenotype and matrix production during in vitro expansion [57–60].

Chitosan

Chitosan is a polysaccharide derived from chitin (found in arthropod exoskeletons) that has been partially or fully deacetylated. It is composed of linear chains of β-linked D-glucosamine residues. Chitosan has been studied as both a scaffold and a controlled delivery system for bioactive factors [61,62]. There is interest in chitosan as a cell delivery

vehicle because it demonstrates good biocompatibility, and some formulations exhibit the property of temperature-dependent gelation, in that they are liquid at room temperature but gel when exposed to physiological temperatures [63]. In addition, the degree of deacetylation of chitosan directly influences the degradation rate of the constructs as well as the host inflammatory response. A lower degree of deacetylation was associated with an increased degradation rate and host inflammatory response. Thermosetting chitosan constructs injected subcutaneously into nude mice supported chondrocyte growth and matrix production, although the constructs were mechanically inferior to native cartilage [62]. Chitosan constructs were also injected into osteochondral defects created in rabbit knees. Retention of the constructs in the defects was observed at 1 week despite full mobility and weight-bearing. BST-CarGel, a chitosan scaffold used in conjunction with microfracture, is in Phase III clinical testing in the United States and has been approved in several other countries including Australia, Canada, and most of Europe. This treatment resulted in improved lesion filling and cartilage repair quality compared with microfracture alone [64,65].

Composite scaffolds using chitosan combined with alginate and/or HA have also been investigated. Li et al. cultured HTB-94 chondrocytes in interconnected 3D porous chitosan−alginate scaffolds and found promoted cell proliferation and enhanced phenotype expression of chondrocytes in these scaffolds compared with chitosan-only scaffolds [66]. Yamane et al. observed higher cell adhesion, proliferation, and aggrecan synthesis in the HA-coated chitosan hybrid polymer fiber sheet than that in the chitosan fiber sheet [67]. Tan et al. developed injectable in situ−forming composite hydrogels consisting of chitosan and HA for cartilage tissue engineering [68]. Hsu et al. evaluated a chitosan−alginate−hyaluronate scaffold modified with a protein containing an arginine-glycine-aspartic acid (RGD)-modified adhesion peptide motif [69]. It was noted that glycosaminoglycan and collagen synthesis was greater in the chitosan−alginate−HA−RGD scaffold compared with chitosan−alginate and chitosan−alginate−HA scaffolds.

Synthetic Scaffolds

Bioscaffolds derived from natural materials are generally considered more favorable in terms of the biological response they can elicit from cells compared with synthetic scaffolds [70]. However, biological materials can be difficult to generate in large quantities with acceptable consistency and often exhibit poor mechanical characteristics [43]. Synthetic materials are generally less expensive than biologics and are created de novo and provide more precise control over the structural properties, mechanical properties, and rates of resorption with a great deal of batch-to-batch consistency [71].

The most common synthetic polymers and traditional tissue engineering scaffolds are polyglycolic acid (PGA), polylactic acid (PLA), polyethylene oxide, and various derivatives and copolymers based on these entities including poly-lactic-co-glycolic acid (PLGA) [43]. These biodegradable polymers have a long history of medical use and are able to be fabricated and processed in a variety of ways [19]. These materials serve as the basis of scaffolds for the adherence, growth, differentiation, and matrix production of chondrocytes or MSCs [72−75]. In general, the materials exhibit many beneficial properties for the production of engineered tissue: a high surface area to volume ratio if processed correctly, sufficient porosity to allow for nutrient and waste diffusion, the potential for surface modification, and the ability to control their degradation rate via selection and modification of their chemical composition [76]. In particular, the ability specifically to control the rate of degradation is important for scaffold survival in vivo. The scaffold must provide sufficient mechanical strength when first implanted, but it should optimally degrade at the same rate as new tissue generation. If degradation is too rapid, there is a risk of cell loss, scaffold failure, and inflammation and the death of surrounding tissue owing to the rapid release of acidic breakdown products [72,77]. Conversely, an overly slow rate of scaffold degradation would likely impede tissue incorporation. The question remains, how do we determine the replacement rate of the cartilage tissue? This depends on a number of factors including the scaffold itself, but more important, the age of the patient and the level of inflammation in the joint. Articular cartilage changes with age, including increased collagen cross-linking, decreased synthesis of type 2 collagen and aggrecan, and reduced tensile strength [78]. Moreover, senescent cells contribute to the poor reparative abilities of aging cartilage. Senescent cells do not divide but instead cause chronic inflammation in the aging tissue [79]. Chondrocytes can also become senescent, slowing down the rate of scaffold replacement with new cartilage, further affecting the rate of new tissue growth.

Synthetic scaffolds have been processed in a variety of configurations from preformed fibers, meshes, and membranes to photopolymerized injectable gels. Preformed solid scaffolds are seeded in vitro by incubation in a cell suspension. These scaffolds may be applied to large, shallow, or open defects. Li et al. manufactured an electrospun nanofibrous PLGA/poly-ε-caprolactone (PCL) amalgam to mimic the natural extracellular architecture of cartilage better [80]. Their study found that a nanofibrous scaffold was more favorable to promoting cell expansion and matrix deposition over microfibrous scaffolds for cartilage tissue engineering [81]. Solid synthetic scaffolds have the

potential to be modified with natural materials to improve biological characteristics. Enhanced cell attachment and proliferation have been achieved in various composite scaffolds combining synthetic polymers with natural materials, including PLA sponge incorporated with cell-seeded alginate [82], PLGA or a poly-L-lactic acid sponge filled with collagen microsponge [83,84], macroporous PLGA scaffold conjugated with HA on the porous surface [85]. Generally, natural materials have difficulty making mechanically strong engineered cartilage with thickness comparable to partial-thickness and full-thickness articular cartilage defects. Chen et al. successfully prepared a unique composite web with adjustable thickness from 0.2 to 8 mm featuring web-like collagen microsponges formed in a mechanically strong knitted PLGA mesh [86].

Achieving adequate cell densities and uniform cell seeding continues to be challenging [72]. A considerable amount of work by our group and others has focused on liquid polymer solutions that are polymerized or cross-linked in situ after incorporating cells and bioactive factors. Such solutions allow the uniform incorporation of cells throughout the scaffold and the development of minimally invasive application techniques [87–89]. Finally, these in situ polymerizable solutions offer the possibility of precise control of the final shape and composition of the scaffold. For example, there has been considerable work by our group and others studying bilayered constructs in which one layer contains MSCs and the other contains chondrocytes, to approximate the cell–cell interactions that would occur in native tissue. Recapitulating the zonal architecture of native cartilage has also been investigated using sequentially photopolymerized hydrogel layers to generate an engineered tissue that more closely approximates normal cartilage [88,90–92].

Novel polymers based on self-assembling synthetic peptides have been studied as a potential scaffold with an internal microstructure closely resembling ECM for cartilage tissue engineering. These peptides spontaneously form hydrogels in response to changes in their environment, such as alterations in pH or ionic strength [93]. The nanofiber structure in these hydrogels is approximately three orders of magnitude smaller than that of most polymer fibers and more closely approximates the structure of native ECM. These materials have the potential for extensive modification by incorporating peptide domains that influence cell adhesion, differentiation, and proliferation [94]. 3D culture of chondrocytes in peptide hydrogels results in the maintenance of chondrocyte phenotype and secretion of cartilage-specific matrix, with increased proliferation and improved mechanical characteristics compared with chondrocytes cultured in agarose [93,95].

Biological Factors

Growth factors, cytokines, protein gradients, cell–cell interactions, and ECM–cell interactions control cellular differentiation, migration, adhesion, and gene expression. How growth factors control cartilage development, maintenance, and changes during diseased states has been investigated intensively. The primary growth factor families that control cartilage homeostasis are the TGF-β superfamily, BMPs within the TGF-β superfamily, insulin-like growth factors (IGFs), and fibroblast growth factors (FGFs). The role of each of these factors in cartilage growth and homeostasis, as well as effects on stem cells, is varied and diverse. TGF-β and IGF generally exert anabolic effects on chondrocytes including inducing increased type II collagen and glycosaminoglycan synthesis, maintaining chondrocyte phenotype, and promoting chondrocytes proliferation, whereas FGF suppresses proteoglycan synthesis and encourages chondrocytes to take a fibroblast morphology [96]. Although TGF-β signaling generally maintains chondrocyte phenotype and encourages chondrocyte growth, TGF-β signaling is altered in OA, resulting in deleterious effects [97]. Both TGF-β and BMP-2 induce human embryonic stem cells to undergo chondrogenic differentiation [98]. Consequently, cell choice will influence the choice of growth factors applied to an engineered cartilage construct, or vice versa. Most growth factor–only related approaches to preventing cartilage loss involve modulating either anabolism or catabolism of the cartilage tissue and/or inhibiting proinflammatory cytokine signaling. Examples include intraarticular injection of TGF-β, IL-1β inhibitors, and matrix metalloproteinase-13 inhibitors [99,100]. However, the diverse roles of growth factors cause difficulty in attempting to implement them in cartilage engineering. For example, BMP-2 causes chondrogenic differentiation in early embryonic distal digit formation, but it causes cell death in a later phase [101,102]. Some anabolic growth factors may cause osteophytes in OA or do not induce a response in older cells, or they may not be enough to combat the degratory enzymes present in a diseased joint [103].There are also additional signaling molecules, integrins, which are molecules that span the cell membrane and connect the cell cytoskeleton to the ECM. This allows the cell environment, including material stiffness and mechanical forces, to influence cell morphology, migration, and signal transmission [104].

Growth factors can also be broadly administered to a cartilage defect via platelet-rich plasma (PRP), an autologous blood product containing platelet-derived growth factors. PRP contains TGF-β1, IGF-1, vascular endothelial

growth factor, and platelet-derived growth factor. PRP can be made with or without leukocytes. The mechanism of how PRP coordinates an antiinflammatory, proliferative, or remodeling response in cartilage is unknown but is thought to result from stimulating cell proliferation, migration, and matrix synthesis [105,106]. Despite the lack of FDA approval, the components that form PRP are approved, and PRP has been used in cartilage defects clinically, in many cases combined with microfracture or scaffolds [107]. There are many proposed mechanism of action for PRP, including that the growth factors secreted by platelets are responsible for at least part of the cartilage repair and pain relief; however, more research is needed to define PRP's mechanism of action further [108]. Once a mechanism is defined, it will be easier to optimize PRP to treat OA.

Biological scaffolds composed solely of tissue ECM and formed into particles also show benefits in reducing OA progression in animal models. Treatment of diseased joints with biological particles exhibited decreased IL-6 and IL-1β and increased type II collagen and aggrecan expression [109,110]. ECM and PRP demonstrate the power in leveraging nature in engineering. Discovering exactly how these therapies work will be pivotal in determining concentrations and ratios of growth factors and ECM components that are needed for cartilage regeneration, which remain unknown. A final consideration for the use of growth factors in engineering cartilage is the difficulty in their administration. Because most growth factors have limited half-lives, it is difficult to achieve therapeutic concentrations at sites of cartilage damage. Gene therapy techniques are being developed to deliver therapeutic genes encoding necessary gene products to cells at the site of cartilage injury to synthesize biological factors of interest for sustained local expression [111]. In contrast to measuring and monitoring growth factor administration, gene transfer provides a local and sustained supply of bioactive proteins. Gene therapy has encountered obstacles with delivery methods; however, upon development of a reliable delivery technique, genetic engineering may interface with tissue engineering [112].

Bioreactors

A fundamental characteristic of many musculoskeletal tissues is their responsiveness to mechanical stimuli [113]. Articular cartilage is subject to complex forces through its range of motion, including shear, compression, and hydrostatic pressure. These forces affect the growth and functioning of chondrocytes via direct cell deformation, alteration of cellular microenvironment, alteration of cell−matrix interactions, and enhanced mass transport within the matrix [30]. Mechanical stimulation influences chondrocyte morphology, biochemistry, and biomechanical and electrochemical properties, including upregulating chondrocyte expression of aggrecan and type II collagen synthesis compared with chondrocytes grown in a static environment [114,115]. These factors imply that developing a transplantable piece of cartilage in vitro will be more successful in a 3D bioreactor than in a 2D culture system, which will be discussed further [116]. High cell densities within a scaffold (on the order of $20-100 \times 10^6$ cells/ml) are also needed to create a transplantable piece of cartilage in vitro [72]. The need for high cell density and 3D culturing can lead to potential problems with nutrient and waste transport, particularly as the constructs get larger and more matrix is deposited. Static culture that relies on passive diffusion of nutrients and waste may be inadequate to serve the needs of metabolically active tissue.

To enhance the biochemical and mechanical properties of engineered cartilage tissues before implantation, bioreactors have been developed to provide adequate mass transfer and mechanical stimulation. Culture of chondrocytes in 3D bioreactors is also beneficial for measuring the effect of mass flow and dynamic mechanical loading on cartilage formation in an isolated manner [117]. Various bioreactor systems have been applied, including rotating-wall vessel, direct perfusion bioreactor, compression bioreactor, and spinner flask [118]. These configurations are illustrated in Fig. 53.2 [119]. Pei et al. cultured bovine chondrocytes in a variety of preformed scaffolds in static conditions and in a rotating bioreactor system [120]. Constructs cultivated in the rotating bioreactor system demonstrated more uniform cell seeding, greater cell numbers, and enhanced chondrogenesis compared with their static counterparts. Raimondi et al. used a novel forced-perfusion bioreactor system to expose the inner portions of their chondrocyte constructs to bulk fluid flow and hydrodynamic stresses, as opposed to rotating bioreactors that expose the surface only to fluid stresses and convective mass transport [121]. The constructs cultivated in this bioreactor also demonstrated greater cell proliferation and better structural integrity than in static conditions. Vunjak-Novakovic et al. demonstrated that dynamic laminar flow patterns on chondrocytes grown on a PGA scaffold resulted in higher fractions of collagen and glycosaminoglycan and improved mechanical and electromechanical properties compared with chondrocytes grown in static culture or turbulent flow conditions [114]. Kisiday et al. used alternating-day mechanostimulation on chondrocytes to increase proteoglycan accumulation [95]. Waldman et al. compared shear with compression stimulation and demonstrated a greater effect on ECM molecule synthesis with shear forces [122]. Increased ECM translates to an increased load-bearing capacity and stiffness by the cartilage

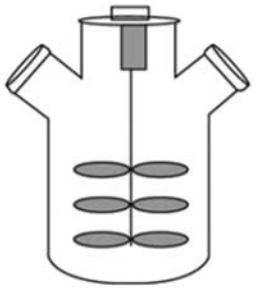
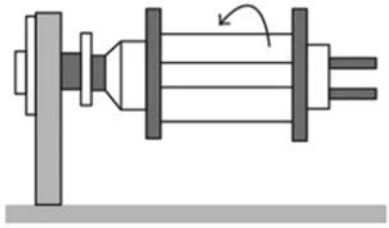
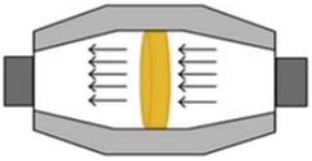
Bioreactor type	General descriptions	Mass transfer mechanism	Shear stress	Special usage	Tissue	Considerations
Static culture	Batch culture with no flow of nutrient	Diffusion (high)	Very low	Cell proliferation	—	Homogeneous structure of cell constructs and nutrient diffusion limitations
Stirred flasks 	Magnetically stirring of medium	Convection (high)	High	Dynamic seeding of scaffolds	Cartilage	Appropriate scaffold and balance between increasing mass transfer and modulating shear stresses
Rotating wall 	Rotating at a speed so the construcsts in the reactor are maintained "stationary" in a state of continuous free fall	Convection (high)	Low	Tissue constructs which need dynamic laminar flow	Cartilage, bone and skin	Operating conditions (e.g., speed of rotating) especially for growing large tissue mass
Perfusion 	Flow of medium over or through a cell population or bed of cells	Convection (moderate) and diffusion (high)	Moderate	Tissues physicochemicaly and environmentally relevant to human tissues	Epithelial cells, intestinal, bone, cartilage, and arteries	Seeding and attachment of human cells especially within the scaffold body

FIGURE 53.2 Comparison of tissue engineering bioreactors. *Reprinted from Salehi-Nik N, Amoabediny G, Pouran B, Tabesh H, Shokrgozar MA, Haghighipour N, et al. Engineering parameters in bioreactor's design: a critical aspect in tissue engineering. BioMed Res Int 2013;2013:762132. Copyright (2013) by Hindawi Publishing Corporation, Open access.*

construct [114,122,123]. Nevertheless, high shear conditions promote apoptosis in chondrocytes, resulting in matrix degradation [124].

Hung et al. demonstrated that mechanical stimulation affected gene expression and biochemical and mechanical properties of bovine articular chondrocytes cultured in agarose. It was shown that the effect was proportional to the frequency of stimulation (which varied between 0.005 and 1 Hz) and was synergistic with TGF-1 and IGF-1 [113]. Benya theorized that dynamic stimulation maintains the chondrocyte's round cell shape, which promotes protein synthesis [32]. Shieh and Athanasiou proposed a number of cellular methods for mechanotransduction [1]: transduction of biochemical signals in chondrocytes by changing physicochemistry including the osmotic pressure, pH, fluid flow, and electric potential of the matrix environment [2]; conformational change of ion pumps and channels [3] via integrins that mediate cell–matrix interaction [4]; nuclear deformation changes in the nuclear pore complex influencing DNA available for transcription; and [5] deformation of the cytoskeleton [125]. Further clarification of the signaling pathways involved in mechanotransduction could yield better design of therapies for cartilage repair.

Additive manufacturing technologies have also reached the field of cartilage tissue engineering, in which 3D printing has been employed to create customizable scaffold designs. These 3D printed scaffolds can be used to grow and differentiate cells for tissue engineering, similar to scaffolds processed with more traditional methods. Pati et al. printed decellularized cartilage matrix seeded with human adipose-derived stem cells onto a PCL framework and found satisfactory cell viability and type 2 collagen expression. In addition, cartilage ECM provided a superior microenvironment for cell proliferation over other printable materials including alginate [126]. Because decellularized ECM maintains its complex microstructure, it is likely to become a popular material for 3D printing combined with synthetic materials to provide improved mechanical properties.

Combining mechanical stimulation of chondrocytes with growth factor administration enhances matrix synthesis to a greater degree than either variable alone. Mauck et al. demonstrated that dynamic deformational loading combined with TGF-1 or IGF-1 increased ECM production in a 3D scaffold [123]. The synergy may be a result of mechanical compression causing increased transport and accessibility of growth factors [125]. In addition, Bhumiratana et al. showed that culturing human MSCs (hMSCs) in a well with the addition of TGF-β enabled the hMSCs to condense into cellular bodies [127]. These cell bodies were induced to fuse and form centimeter-sized, mechanically functional cartilage that fused and integrated with bone scaffold. These mesenchymal cell bodies were also tested for their ability to fill a cartilage defect, in which they fully integrated with the existing cartilage owing to collagen II and aggrecan. Moreover, Eng et al. constructed intricate 3D hydrogels designed to predict chemotactic directions of cell migration in MSC and endothelial cell cocultures [128]. Just as sequential administration of growth factors increases chondrocyte proliferation, the investigation of mechanical stimulation and growth factor administration timing could yield future advancements in cartilage engineering [129].

Translation of Cartilage Tissue Engineering

Preclinical Translation

There are many important aspects to consider in designing preclinical ("in vivo") studies for translating engineered scaffolds/cell/peptide therapies, including the clinical indication, animal, and disease model. The type of therapy will also dictate the study design and approach. For example, initial studies for cell-seeded scaffolds often start with simple subcutaneous implantation to examine their ability to generate cartilaginous tissue in vivo. If these studies show that the in vivo environment and tissue scaffold are not hostile to the development of cartilage and osteochondral tissues, the therapy is then tested in the target tissue to determine therapeutic efficacy.

The choice of animal model and the application of the material are crucial to moving therapies to the clinic successfully. For example, if an engineered cartilage construct is intended to fill articular cartilage defects, it is likely that the person receiving the cartilage construct will have OA or some chronic inflammation. Thus, not only is the promotion of new cartilage formation and lack of rejection by the animal important, because the presence of disease will have many implications for the success of the therapy, preclinical testing of materials needs to take into account the disease state. In the case of OA, there are several mouse as well as rat and guinea pig models of OA requiring mechanical loading of the knee joint, transecting the ACL or meniscus, or injecting monoiodoacetate to induce cartilage damage [130].

Animal models still have room to improve. Many therapies, such as *inducible nitric oxide synthase* inhibitors and COX inhibitors, were shown to decrease cartilage lesions and osteophytes in separate OA animal models but did not show clinical efficacy in humans [131]. This problem may be partly because most preclinical testing is performed early in OA disease progression; however, people treated clinically are in late-stage OA. Also, animals used experimentally are generally young adults as opposed to most patients with OA, who are adults aged over 60 years [131]. Studies evaluating the biological differences in OA between young and old animals are needed to advance the utility of animal models in screening therapies.

Clinical Translation

There are many different cartilage bioengineering therapies in clinical testing. Most therapies consist of scaffold alone or scaffold plus cells (MSCs or chondrocytes), MSC-only therapies, PRP injections, and viscosupplementation [132]. ACI is meant for localized cartilage injuries and applies the patient's own expanded chondrocytes in solution to the defect under a surgically closed periosteal flap [16,17]. This allows the autologous articular chondrocytes to synthesize new cartilaginous matrix in the defect site. ACI is clinically approved (Carticel), but the clinical outcomes of the standard ACI methods have disadvantages including donor-site morbidity, risk for leakage of transplanted chondrocytes, complexity of the surgical procedure [133], uneven distribution of the cell suspension in the transplanted site [134], periosteal hypertrophy [135], and dedifferentiation of the chondrocyte phenotype during

in vitro monolayer culture [32,136]. These problems prompted matrix-induced ACI (MACI), second-generation ACI to be developed. MACI involves applying cell-seeded constructs instead of cell suspensions for cartilage repair. Vericel MACI therapy has been approved clinically by the FDA and is the first autologous cellularized scaffold approved in the United States for the repair of cartilage defects. This MACI technique uses an implant with a bioresorbable type I/III collagen membrane with approximately 1,000,000 cells/cm^2. This therapy has been found to improve the Knee Injury and Osteoarthritis Outcome Score more significantly than microfracture but to have similar histological repair outcomes in Phase III clinical testing [137].

Variations of the MACI approach include a technology in which the in vitro chondrocyte expansion was performed using a 3D scaffold made from modified HA [138]. The scaffold was then implanted into cartilage defects via a miniarthrotomy or arthroscopic approach. Their cohort includes 141 patients observed for 2−5 years. Their results appear impressive, with improvement in subjective symptoms reported in over 90% of patients. Second-look arthroscopy was performed in 55 patients, and the cartilage repair was graded as normal or near-normal in over 95% of these patients. Biopsies were taken in 22 of these 55 patients, which revealed a hyaline appearance in 12 of 22; the remainder were a mixed or fibrocartilaginous appearance. Histogenics has the most advanced engineered cartilage therapy in the United States. This MACI therapy, NeoCart, expands chondrocytes, after which they are seeded on a collagen scaffold that is matured in a bioreactor before implantation in a cartilage defect. In Phase III clinical testing in the United States, results demonstrate reduced pain, increased function, and imaging-confirmed defect filling with NeoCart treatment [139].

Biomaterials alone are also being implanted in conjunction with microfracture or other autologous cell or tissue sources for focal cartilage repair to provide a simplified off-the-shelf therapeutic. Various approaches have been developed to incorporate biomaterials with microfracture, e.g., using polymer scaffold combined with minced cartilage from a biopsy [140,141], implanting collagen membranes microfracture [142], and applying chitosan mixed with blood after microfracture [143,144]. These biomaterial-guided tissue repair methods may be more economical and provide an off-the-shelf therapy that is more efficacious than surgical intervention alone.

Achieving integration of engineered tissue with host cartilage is still a troublesome problem for cartilage reconstruction, especially for long-term cartilage repair [145]. Cartilage integration failure was common and probably caused by a variety of factors, including limited chondrocyte mobility in the cartilage extracellular matrix, chondrocyte cell death at the wound edge, chondrocyte dedifferentiation in the engineered tissue, the type of biomaterial scaffold, and the origin and stage of the cells used for cartilage tissue engineering. Corresponding solutions have been reported to enhance the construct, including cartilage integration by pretreating the cartilage interface enzymatically to break down collagenous matrix [146,147], inhibiting the chondrocyte death at the lesion edge [148], and using immature constructs instead of mature constructs [146]. We developed a mechanically strong biological glue to bridge native cartilage with biomaterial scaffolds [149]. This glue is based on chondroitin sulfate, a major component of the cartilage ECM, functionalized with methacrylate and aldehyde groups to react chemically with the biomaterials and cartilage proteins. Using this glue, full integration was achieved in full-thickness chondral defects after marrow stimulation. Studies suggest that PRP can improve cartilage integration of explants [150,151] and that immunomodulation has an important role in successful explant integration.

CURRENT AND FUTURE TRENDS IN CARTILAGE ENGINEERING

These tissue engineering techniques show great potential advantages over conventional surgical options and are being applied to clinical practice intensively [152]. The ultimate aim of articular cartilage tissue engineering is to design an engineered tissue that can regenerate to hyaline cartilage with normal knee functions and integrate fully with the surrounding native cartilage. To date, no engineered tissue construct fulfills this criterion; thus, there is considerable ongoing work in various aspects of cartilage tissue engineering research from cell type to bioscaffold, biological factor, bioreactor, and tissue translation. It would be nearly impossible to summarize the vast body of this research in a single chapter, so the range of studies outlined here is necessarily only a brief summary of the past and current literature on selected topics.

The recurring theme throughout much of the current literature is that the engineered tissue has the histological appearance and biochemical makeup of cartilage of varying stages of maturation. However, it has been reported that mechanically, most of these constructs are inferior to native cartilage. As the basic techniques of chondrocyte, osteoblast, and MSC culture are elucidated, the focus is shifting toward improving the mechanical properties of engineered tissues. One concern is that, to achieve complete reconstruction of cartilage defects, the transplanted tissue can initially have mechanical strength inferior to native cartilage temporally to allow the tissue to mature

and integrate to the surrounding cartilage ultimately under the in vivo environment. A large gap should be considered to exist between in vivo studies and in vitro testing and optimization for the clinical translation of engineered cartilage. In vitro methods should be standardized to provide clear results to develop successful clinical applications for cartilage tissue engineering [35]. Several important basic questions that remain to be answered: What are the optimal types, amounts, and timing of the growth factor milieu? Perhaps PRP will answer this question after further characterization. Will small molecular drugs work effectively for cartilage tissue engineering, because many biological factors are complex and exhibit delivery problems? In vitro models provide the isolated environment necessary to define genetic programming clearly and signaling pathways that are involved in chondrocyte maintenance fail to compensate for all the dysregulated signaling pathways in vivo, which will ultimately dictate the success or failure of a bioengineered therapy. More preclinical models of cartilage defects need to be used to determine the impact of the host environment on bioengineered therapies.

Other areas of intense scrutiny include exploring ways to implement complex tissue elements such as spatial organization and vasculature in engineered tissues. Work on osteochondral tissue generation has been discussed here. However, as the effects of 3D organization of tissues become more fully appreciated, there will be a need to regenerate those structures for the purposes of controlled laboratory study and clinical application. With the use of novel polymers that form hydrogels under controlled conditions, there is the potential for fine control of the shape of engineered tissue as well as the 3D spatial arrangement of heterogeneous cell populations within the scaffold. This has already been demonstrated using photolithographic methods to control hydrogel configuration and cellular organization [153]. A clinical application was demonstrated by Naumann et al., in which computer-aided design techniques were used to fashion an HA scaffold seeded with chondrocytes into the shape of a human ear. The construct demonstrated an acceptable shape as well as evidence of cartilage production in vitro, but mechanical properties were not tested [154]. Another clinical application of computer-assisted arthroplasty was shown by Sidler et al., in which a bony defect was made in the talus of a human cadaver ankle joint. The defect was then analyzed by computed tomography and an implant was fashioned using a computer-aided manufacturing device [155].

An overlooked aspect to cartilage engineering is the use of cytokines and manipulation of the immune cells that produce them. The immune system has typically been viewed as being responsible only for material rejection and tissue destruction, with immune suppression considered ideal. However, in light of work showing the importance of the innate and adaptive immune system in tissue repair, future work will most likely focus on modulating the immune system, not simply suppressing it.

OA treatments such as nonsteroidal antiinflammatory drugs and steroids suppress the immune system and are widely used to reduce pain. However, neither reduces disease progression, which suggests that suppressing the immune system does not improve OA outcome. Harnessing macrophages and T cells for tissue regeneration would be a logical strategy, considering that they already have many functions in tissue remodeling. In addition, because T cell therapies are already used in other diseases such as cancer immunotherapy, they could be adapted for use in cartilage regeneration to guide macrophage polarization and direct the tissue repair process.

Of course, many more questions and challenges remain before the promise of tissue engineering is fully realized. The contributions of scientists in fields as diverse as cell and molecular biology, materials science, chemistry, and mathematics will be required to answer these questions.

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CHAPTER

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Stem Cell Therapy for Musculoskeletal Diseases

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INTRODUCTION

Musculoskeletal Diseases: A Need for Improved Therapies

Disorders of the musculoskeletal system constitute the most common cause of disability in the developed world and account for 25% of all operating room procedures, the largest percentage by organ system [1]. Musculoskeletal disorders can be caused by acute trauma (e.g., fractures, sports injuries), tissue degeneration (e.g., osteoarthritis, spinal stenosis), genetic aberrancies (e.g., muscular dystrophy), and autoimmunity (e.g., rheumatoid arthritis), with frequent interaction among these etiologies. Joint and back pain, often the result of degenerative osteoarthritic changes, together represent the most frequent cause for a physician visit. Whereas total joint replacement of arthritic hips and knees is a remarkably effective treatment to alleviate pain and improve function, there are fewer treatment options for other chronic musculoskeletal disorders. Furthermore, the population is increasingly engaged in physical activities (notably sports) that increase the risk of orthopedic injury. Even if successfully treated to permit return to physical activity, these injuries often predispose the patient to early-onset osteoarthritis. However, given the finite life span of joint prostheses, surgeons are reluctant to offer total joint replacement to a younger patient. Similarly, a myriad of other musculoskeletal disorders have proven recalcitrant to surgical intervention, no matter how sophisticated the surgical technique. For these reasons, it has been the hope of patients, clinicians, and scientists alike that the relatively new disciplines of tissue engineering and regenerative medicine might provide solutions to these formidable challenges of musculoskeletal disorders.

Perhaps no aspect of orthopedic regenerative medicine has been more promising, and equally disappointing, as stem cell therapies for musculoskeletal diseases. That is, although an ever-increasing pace of basic research is developing novel stem cell–based therapies, with promising results consistently found in animal models, relatively few stem cell therapies have been applied clinically. Where they have, the subject number has been small and the study quality has been poor, limiting conclusions regarding the efficacy of these putative therapies. As discussed subsequently, the slow pace of translating stem cell therapies from the bench to bedside is partly the result of financial and regulatory challenges.

Regulatory and Financial Challenges to Stem Cell Therapies

Cell-based therapies represent a relatively new technology and their regulatory purview is still being determined. The US Food and Drug Administration (FDA) issued a set of regulations (21 CFR 1271) in 2001 governing cellular and tissue-based therapies. The underlying principle of these guidelines was that cellular and tissue therapies that require minimal to no manipulation of autologous tissues do not require FDA approval, whereas those that involve any significant modification or treatment, including genetic manipulation, require it. Despite the subsequent issuance of a series of guidance documents, FDA approval necessitates substantial time, effort, and financial investment for the performance of the clinical trials needed to demonstrate safety and efficacy and has effectively limited the implementation of cellular or tissue-based therapies to those with minimal manipulation of autologous

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cells and tissues. Allograft tissues, which contain allogeneic cells, have been implemented in clinical practice by maintaining their classification as transplant tissues that are subject to different regulations. There is ongoing debate about whether mesenchymal stem cells (MSCs) qualify as biologic drugs subject to FDA approval or human cellular or tissue products. Along these lines, in February, 2014, The US Court of Appeals for the District of Columbia Circuit upheld a 2012 ruling that a patient's cells for therapeutic use fall under the aegis of the FDA. The appeals case involved the company Regenerative Sciences and their Regenexx procedure, which involved the use of autologous cells to treat musculoskeletal pathologies.

Most insurance companies have been reluctant to cover the cost of cell-based therapies for musculoskeletal pathologies because outcome studies have been small in number and often of insufficient quality. However, this has not deterred patients from spending up to $10,000 out-of-pocket per treatment for "stem cell" therapies. The success of cell-based therapies with more extensive cellular manipulation, such as genetic engineering, in areas with greater room for dramatic improvement of clinical outcomes, such as orphan diseases of inborn metabolism and cancer, may clear the path for implementing more sophisticated cellular therapies in musculoskeletal pathologies. That is, demonstrated safety and efficacy in treating grave diseases with high morbidity and mortality may ironically facilitate broader implementation of related cell-based therapies for more prevalent, but often less dire, musculoskeletal disorders. On the other hand, promising preclinical studies as reviewed subsequently, demonstrate safety and efficacy of stem cell–based therapies, which it is hoped will prompt clinicians and researchers to partner with regulatory administrators to seek broader application of these technologies to treat human diseases.

Chapter Overview

This review focuses on stem cell therapies for musculoskeletal diseases. In particular, we highlight stem cell applications for individual tissues of the musculoskeletal system (bone, articular cartilage, tendon and ligament, meniscus, intervertebral disc, and skeletal muscle) as well as transitions between tissues (osteochondral interfaces and tendon–bone interfaces). For each, the relevant structure and function are described. Because embryological development of these tissues and the phenotypic characteristics of their resident cells are described in detail in other chapters, only a succinct discussion of tissue-specific endogenous cells is included. There is emphasis on findings of preclinical and clinical studies when possible. However, a comprehensive review of all available stem cell therapies for a given tissue or disease is beyond the scope of this chapter; readers are referred to more extensive reviews for further detail.

STEM CELL THERAPIES FOR MUSCULOSKELETAL DISEASES

Cell-based therapies include numerous cell phenotypes, including adult differentiated cells (e.g., chondrocytes), progenitor cells (e.g., satellite cells, SCs), multipotent stem cells (e.g., MSCs), and pluripotent stem cells (e.g., embryonic stem cells [ESCs], induced pluripotent stem cells [iPSCs]). For all phenotypes, allogeneic, autologous, and xenogeneic cells may be considered. Although adult differentiated cells ostensibly possess the greatest homology to the endogenous cells of the target tissues, autologous cell isolation is associated with the creation of an iatrogenic defect with associated donor site morbidity. Furthermore, these cells often possess a limited expansion potential ex vivo and frequently undergo dedifferentiation during the process [2]. On the other hand, pluripotent stem cells, including ESCs and iPSCs, present several ethical (especially for ESCs) and technical challenges (i.e., need for feeder cells) that have limited their broader use in regenerative applications. Therefore, the MSC has been the workhorse for most investigations of stem cell therapies for musculoskeletal diseases. Although often described as a single-cell phenotype with particular cell surface markers, the term "MSC" is used loosely in the literature to include a multipotent, plastic-adherent population of cells capable of forming colonies. Although MSCs were originally thought to aid tissue regeneration by synthesizing the extracellular matrix (ECM) that would constitute new musculoskeletal tissues [3], studies showing diminished MSC viability and retention at musculoskeletal lesions over time (despite improved healing) suggested that MSCs might also function by secreting paracrine factors [4]. The phenotype and function of MSCs are still an area of active investigation, but as detailed in other chapters, their utility for enhancing healing of musculoskeletal tissues has been demonstrated in preclinical studies, as described subsequently.

Bone

Bone diseases can be caused by different factors, including nutrient deficiencies, genetic alterations, and vasculature disorders, causing bone loss or decreased density, with resulting reduction in bone strength. Congenital bone defects, fracture malunion or nonunion, osteogenesis imperfecta, bone resection caused by osteosarcoma or tumor metastasis, osteoporosis, osteonecrosis, and osteomyelitis are just a handful of musculoskeletal problems affecting bone. Moreover, alterations in bone vascular supply have a critical role in increased susceptibility to osteoporosis, osteonecrosis, and osteomyelitis. These multifactorial pathologies are concerning, especially in the context of an aging population and increased incidence of trauma-related injuries. When the innate regenerative capacity of bone is compromised, as is often the case in these pathologies, there are few biological options to promote new bone growth. Bone autografts are still considered the current reference standard, but they have limitations in terms of size of the defect to be grafted and the need for a second surgical site for the bone harvesting, often the iliac crest of the pelvis. Autografting increases patient morbidity, including increased postoperative pain and risk for infection. Allografts avoid the pitfalls of autografts but do not possess the same strength and cellular component. Because current treatments do not provide optimal outcomes, tissue engineering and regenerative medicine strategies, which use stem cells often in combination with biodegradable scaffolds, represent a possible alternative.

The successful development of clinically relevant engineered bone relies largely on the choice of stem cells, which are the main players for the actual production of the bone tissue. Another critical component is the scaffold, which should possess osteoconductive, osteoinductive, and sufficiently strong mechanical properties, as well as the ability to integrate with the host vascular network. Main vascularization strategies have included the delivery of angiogenic factors within with three-dimensional (3D) scaffolds, in vitro prevascularization strategies, and the use of coculture systems [5].

Several materials have been employed as stem cell carriers for engineered bone. Calcium phosphates such hydroxyapatite (HAp) and β-tricalcium phosphate are known for their osteoconductive properties and stiffness. Bioactive glasses are another class of inorganic materials exploited in bone engineering, owing to their ability to support bone formation in vivo. Apart from inorganic matrices, natural and synthetic polymers have been extensively used for bone engineering. Biopolymers (e.g., collagen, silk, chitosan, polyhydroxyalkanoates) offer low manufacturing/disposal costs, can be obtained from renewable resources, and provide natural cues for cell signaling, adhesion, and remodeling. On the other hand, synthetic polymers (e.g., polyglycolic acid [PGA], poly-lactic acid [PLA], poly-ε-caprolactone, biodegradable polyurethanes) can be manufactured and tailored with desired physiochemical and degradation properties. Biological scaffolds can be prepared by decellularizing bone or specific ECM preparations.

Biologically inspired scaffolds contain signals that can induce the simultaneous development of bone and vasculature within the construct. Technological advancements in the rapid prototyping and additive manufacturing field have given researchers powerful tools to model these materials in various shapes to address clinically relevant issues [6]. Although osteoinductivity and osteoconductivity usually depend on a material's chemical nature, these technologies allowed the development of scaffolds with controlled ultrastructure (e.g., interconnected macroporosity for optimal osteoblast differentiation and vasculature development) as well as stiffness matching the mechanical properties of native bone. A novel fabrication technology that is gaining relevance for bone tissue engineering is bioprinting, which consists of the precise deposition of cell-laden biomaterials (referred to as bioinks) in predefined 3D structures. The use of computer-aided design and manufacturing allows for the production of specific constructs exactly matching the dimensions of the target defect. Vasculature can be bioprinted, too; for example, it can be used to dispense smooth muscle cells, endothelial cells, and fibroblasts as single- and double-layered vascular tubes.

The design of a stem cell–based technology for engineering bone requires the selection of suitable cell sources, which should possess self-renewing capacity and ease of maintenance and manipulation in vitro for subsequent implantation into the defect or lesion. One benchmarked cell source for bone tissue engineering is the MSC, owing to its well-defined capacity to undergo osteogenic differentiation. Together with bone marrow–derived MSCs (BM-MSCs), adipose-derived MSCs (ASCs) are an accepted cell source for engineered bone. MSCs have been thoroughly investigated, in combination with several types of scaffolds, in preclinical settings using small animal models such as mice [7], as well as critical-size defects in rabbits [8] and in more relevant ovine models [9,10].

An example of the clinical use of MSCs in bone engineering was documented in a pilot study treating five patients with unilateral cleft lip and palate. MSCs were used in combination with a commercially available collagen and HAp scaffold (Geistlich Bio-Oss), showing bone formation spanning the previous alveolar cleft

after 6 months in all patients. Despite this early clinical success, myriad investigations suggested that improved fabrication of biomimetic engineered bone is augmented by the use of endothelial stem cells. Blood-derived endothelial progenitor cells are a good candidate for this aim, and they have been used in cotransplantation studies with MSCs to improve neovascularization and bone regeneration in rats [11]. iPSCs can also be identified as cell sources for bone tissue engineering because they can be differentiated into osteoblasts and osteoclasts capable of producing mature bone–like tissue both in vitro and in vivo [12,13]. iPSCs have also been shown to form vasculature [14].

To optimize cell seeding and mass transport within a scaffold, bioreactor technology is also being pursued. Several setups have been developed in the past years, such as spinner flasks, rotating wall vessels, and perfusion bioreactors. Although these systems provide fine control over the experimental parameters, they often fail to produce sufficient quantity and quality of bone; hence "in vivo bioreactor" technologies have been developed to produce clinically relevant volumes of vascularized bone. These techniques rely on the ectopic cultivation, usually in a periosteal flap, of sizeable bone grafts, exploiting the body's regenerative capacity as a living bioreactor to cultivate stem cells, scaffolds, and growth factors. Once sufficiently matured, these functional bone grafts then can be implanted into a defect.

Despite promising results in preclinical application of MSCs for bone regeneration, their clinical use remains nascent and their effects need to be investigated further. Increased therapeutic applications of stem cells for enhanced bone repair will come with elucidating the complex interactions among all of the cellular components of bone. Moreover, bone tissue engineering would be upgraded by automation and standardization, increasing the likelihood of delivering an effective and economically viable technology.

Articular Cartilage

Three types of cartilage exist in human body, including hyaline cartilage, elastic cartilage, and fibrocartilage. In these tissues, glycosaminoglycan (GAG) binds to protein fibrils to form a highly hydrated matrix that is flexible and resilient to bearing. In hyaline cartilage, the fibrils are mainly formed from collagen type II, compared with an elastin–collagen type II mixture in elastic cartilage and collagen type I/II in fibrocartilage. Hyaline cartilage exists in the nose, surfaces of long bone ends, the rib tips, and the rings of the trachea. The cartilage in the epiglottis and pinnae shaping the outer ear are elastic cartilage. The meniscus and temporomandibular joint are typical fibrocartilage. In this chapter, we will primarily focus on cell therapies for the repair of articular cartilage (AC) defects.

AC is the smooth surface that covers bone ends, allowing frictionless movement as well as load bearing. Under physiological conditions, AC has a glassy appearance, thus its name (*hyalos* is Greek for "glassy"). The avascular and aneural nature of cartilage prevents the tissue from receiving sufficient nutrients and reparative cells necessary for wound healing, which limits AC self-repair [15]. For example, a study reviewing knee arthroscopy results reported that the incidence of chondral defects was higher than 60%, although many patients did not report pain or compromised mobility [16–19]. In a review of 993 consecutive knee arthroscopies, 11% of knees were found to have one or multiple full-thickness cartilage lesions (International Cartilage Repair Society Grades 3 and 4) [17]. In a systematic review of multiple databases, the prevalence of full-thickness focal chondral defects in athletes was strikingly higher than that of nonathletes (36% versus 11%) [20].

No treatments are available to regenerate stable hyaline cartilage completely in a chondral defect. In addition to conventional palliative treatments, some surgical protocols such as microfracture and autologous chondral transplantation have been shown to result in filling of the lesions, with associated pain reduction and improved cartilage function. However, critical challenges remain. For small cartilage defects, the microfracture technique is commonly employed to induce bleeding and allow migration of reparative stem cells and cytokines from the bone marrow [21,22], which has been shown to improve joint functionality and pain relief for over half of patients when applied in those who are young. However, the cartilage formed is usually fibrocartilage, which eventually fails owing to structural and mechanical properties inferior to those of hyaline cartilage. Thus, alternative regenerative techniques are being developed to improve microfracture.

Cell-based therapies, such as autologous chondrocyte implantation (ACI), have shown the most promise for the regenerative repair of cartilage [23]. ACI is a two-stage procedure involving chondrocyte isolation and in vitro expansion, followed by implantation [24]. ACI is the only FDA-approved cell-based therapy for focal cartilage defect repair (marketed as Carticel by Genzyme Biosurgery). However, harvesting of autologous cartilage results in donor site morbidity, and the chondrocyte phenotype is reduced or even lost upon culture expansion in vitro, thus

impairing or compromising the outcome [25]. Consequently, it remains uncertain whether ACI provides a superior outcome in cartilage repair over other treatments such as microfracture [26,27].

As alternative cell sources for cartilage regeneration, ESCs present ethical issues and potential immunorejection. iPSCs are often excluded owing to safety concerns, notably tumorigenicity. Therefore, the use of autologous MSCs continues to receive the most attention because of their relatively easy access and efficient chondrogenesis upon stimulation [28]. There are two major avenues to using MSCs for the treatment of chondral defects: the differentiation of MSCs into chondrocytes and the application of factors produced by MSCs.

Adult tissue-derived, multipotent MSCs have the ability to differentiate into chondrocyte-like cells and have been considered a promising candidate cell type to replace chondrocytes for cartilage tissue engineering [22,29]. For example, direct intraarticular injection of MSCs showed improvement in cartilage healing and regeneration [30,31]. In our studies [32–34] as well as those of others, the reparative potential of ASCs was well-demonstrated in cartilage regeneration.

To become chondrocytes, MSCs must undergo a chondroinductive process. Embryonic chondrogenesis in the developing limb bud begins with mesenchymal cell recruitment, proliferation, and condensation. In this sequential process, cell condensation is a critical step that is initiated by several growth factors, including transforming growth factor-β (TGFβ), fibroblast growth factor (FGF), Wnt, and bone morphogenetic proteins (BMPs), acting in concert [35–37]. In particular, TGFβs and BMPs are the most potent inducers of MSC chondrogenesis, characterized by the enhanced expression of SOX9, collagen type II (COL2) and aggrecan and the deposition of collagen and proteoglycans rich in sulfated GAGs. For human MSCs, TGFβ2 and TGFβ3 were shown to be more active than TGFβ1 in promoting chondrogenesis [38]. In fact, the TGFβ family is the most used to direct MSC chondrogenesis. In addition to soluble factors, a variety of both natural and synthetic scaffolds have been used to serve as carriers for the cells or to provide a chondrosupportive microenvironment [39].

Interestingly, in cultures of MSCs undergoing chondrogenesis in vitro, the expression of chondrocyte hypertrophy-associated genes has been widely reported. Hypertrophy refers to the process seen in endochondral ossification of growth plate cartilage, in which chondrocytes undergo enlargement, glycolytic metabolism, altered matrix production, and eventual matrix mineralization [40]. These activities are also associated with osteoarthritis chondrocytes [40,41]. Hypertrophy is characterized by an upregulation in the expression of COL10, specific matrix metalloproteinases, and vascular endothelial growth factor (VEGF), and an increase in alkaline phosphatase activity, which are regulated by the Runt-related transcription factor 2 (Runx2) transcription factor, a marker typically associated with osteogenic differentiation [42]. The ECM deposited by hypertrophic chondrocytes is structurally inferior to that of hyaline chondrocytes, eventually leading to poor cartilage regeneration or formation of nonhyaline cartilage [43,44]. Hypertrophy is not related to the tissue source of the MSCs; it is observed in adult MSCs from all origins. These observations point to the deficiency of currently practiced methods of MSC chondroinduction. Hence, the major challenge of applying MSCs for hyaline cartilage repair is to produce stable articular-like, hyaline chondrocytes that are resistant to hypertrophic conversion and subsequent ossification.

The mechanism of the abnormal hypertrophy is not completely understood. It may result from inherent chondro-hypertrophic and osteogenic tendencies of MSCs, because the insufficiency of current formulas in controlling the hypertrophic process is observed in adult MSCs from all tissue sources. Chen et al. well-summarized the pathways associated with chondrocyte hypertrophy and strategies to inhibit such an unfavorable phenotype [45]. In particular, Wnt/β-catenin signaling was proposed to be associated with hypertrophy, because blocking DKK1 and FRZB, endogenous Wnt inhibitors, increases hypertrophic differentiation. Cao et al. explored the effects of xanthotoxin on MSC hypertrophy and found that xanthotoxin reduced the activation of p38 mitogen-activated protein kinase, leading to the increased expression of histone deacetylase 4, which finally suppressed Runx2 and the expression of hypertrophic marker genes [46]. The PI3K–Bapx1 pathway and Notch signaling have also been shown to be involved in the hypertrophy process [47]. Interestingly, coculture of MSCs with articular chondrocytes may also suppress hypertrophy [48,49]. However, this method would involve the isolation and expansion of (autologous) primary chondrocytes, which is thus hampered by the same limitations as ACI, described earlier.

Eighteen clinical trials using live MSCs to treat cartilage defects are registered at ClinicalTrials.gov. However, few of the completed studies reported results. In a clinical study employing 14 patients, autologous MSCs were used to treat full-thickness chondral lesions of the knee greater than 2 cm^2, with ACI as the control. At all follow-up intervals of up to 24 months, patients treated with MSCs displayed significantly better functional outcomes than those treated with ACI ($P < .05$), including greater range of motion and straight leg raise strength. Subjective subscale scores for pain, activities of daily living, and osteoarthritis outcome scores ($P < .05$) in the MSC-treated group were also superior to those with ACI. No adverse effects from the implants were observed in either group [50].

The safety of autologous MSC implantation was further confirmed in other 2-year follow-up studies [51,52]. To enhance the reparative outcome, multiple cell injections have also been tried. Three intraarticular injections, each containing 20×10^6 MSCs, were performed 4–6 weeks after femoroacetabular impingement. Results showed that modified Harris Hip, Western Ontario McMaster University Osteoarthritis Index, and Vail scores increased from 64.3, 73, and 56.5 at the beginning, to 91, 97 and 83, respectively, at month 24 ($P < .05$). Around 20% of patients reported pain after cell injection [53]. MSC injection has been combined with other surgical methods. For example, after arthroscopic subchondral drilling into grade 3 and 4 chondral lesions, Saw et al. [54] injected hyaluronic acid together with autologous peripheral blood stem cells and found that such combined treatment resulted in hyaline cartilage formation with enhanced quality compared with cell-free treatment.

In addition to their potential to generate chondrocytes, there is substantial evidence to suggest that MSCs produce a wide range of secreted factors that influence the injured tissue microenvironment and orchestrate tissue repair processes. In fact, in many cases in which the positive effects of MSC injection have been observed, the transplanted MSCs are not found at the lesion site at follow-up [4,55]. Therefore, against the initial assumption, increasing evidence suggests that the main therapeutic function of MSCs may not be to replace tissues cells; rather, they may have paracrine effects. As such, the use of MSC secretome, which includes soluble factors as well as extracellular vesicles (EVs) [56,57], has been proposed as a safer and more advantageous approach compared with live MSC-based therapy.

EVs, as well as exosomes, have been applied in many clinical conditions [58], but there are few studies on EVs in the treatment of chondral defects. Although MSC EVs have been shown to have positive effects in treating artificial injury or insult-initiated (secondary) animal osteoarthritis models [59–67], most functions of exosomes are associated with their immunomodulatory properties. In a study using chondrocytes from rats, exosomes from immortalized embryonic day 1-myc 16.3 human ESC–derived MSCs not only promoted chondrocyte proliferation but also enhanced chondrogenic gene expression, as well as GAG deposition [61]. After being incorporated into an acellular tissue patch, stem cell–derived exosomes were able to integrate with native articular cartilage tissues and promoted cell migration at the defect sites, ultimately leading to enhanced tissue regeneration [59]. A study further indicated that such chondrosupportive function may be caused by the high levels of sphingosine-1-phosphate (S1P), which activated S1P receptor 1 on chondrocytes. Blocking S1P with a neutralizing antibody depleted the beneficial effects of EVs in promoting cartilage formation in vivo [68].

Osteochondral Tissue

Defects of both AC and the underlying subchondral bone are often associated with joint pain and instability, risk factors for the development of osteoarthritis. The hallmark of osteoarthritis has been traditionally defined as the progressive degeneration of cartilage; however, several studies reported that subchondral bone is critical in the pathogenesis of osteoarthritis. The continuous mechanical and functional interaction of articular cartilage with the subchondral bone during movement creates reciprocal effects between the two tissues. In fact, antiresorptive drugs that limit bone remodeling have a positive effect on cartilage, whereas antidegradative treatment on cartilage alleviates bone remodeling. During the pathogenesis of osteoarthritis, communication between bone and cartilage is significantly increased owing to vascular infiltration into the cartilage, formation of microcracks and microchannels from bone and bone marrow reaching the cartilage, fragmented tidemark, and fissures. Increased vascularization has been indicated as another hallmark of osteoarthritis, revealing VEGF as an important player in osteoarthritis pathogenesis, because its expression is elevated in arthritic chondrocytes compared with healthy chondrocytes. Treatments of osteoarthritis are mostly symptomatic and based on pain and antiinflammatory medications. Because of the degenerative and chronic nature of this disease, total joint replacement is ultimately necessary in most cases.

Morphological and functional differences of the two main components of the osteochondral complex have always challenged researchers in the development of tissue engineering constructs for the regeneration of this interphase. Most studies have been traditionally focused on the production of gradient structures with different mechanical and chemical properties. Hydrogels are most frequently used for cartilage engineering, owing to the highly hydrated nature of cartilage ECM, whereas stiffer scaffolds are needed to match native bone mechanical properties. A comprehensive review by Nooeaid et al. [69] describes the main materials and scaffolds used for osteochondral regeneration.

The choice of stem cells for osteochondral tissue engineering is also challenging, because bone and cartilage cells are specialized and are characterized by different biological properties. In this case, MSCs are valuable, owing to their ability to differentiate into both cartilaginous and osseous lineages. However, as stated in the earlier section on Bone, engineered bone must also contain an endothelial component to satisfy a clinically relevant engineered

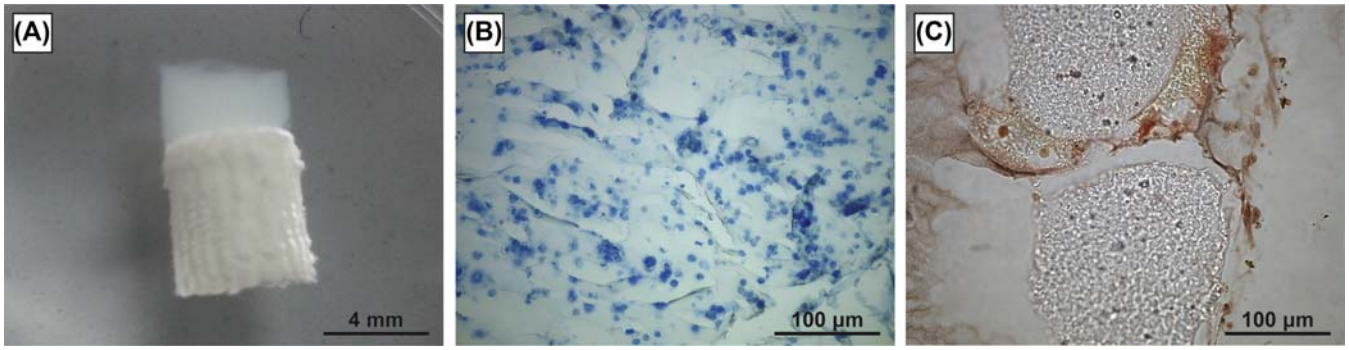

FIGURE 54.1 Engineered osteochondral interphase obtained using a dual-chamber perfusion bioreactor. (A) Macroscopic image of the construct showing photocrosslinked gelatin methacrylate as the cartilage tissue (*top*) and an additively manufactured poly(ε-caprolactone) scaffold as the subchondral bone (*bottom*): both compartments were seeded with human mesenchymal stem cells (hMSCs) and cells were separately yet simultaneously differentiated using two different media streams. (B) Alcian blue histological staining of the chondral tissue showing glycosaminoglycan production by hMSCs. (C) Alizarin red histological staining showing calcium depositions by hMSCs in the osseous tissue.

construct for regeneration in vivo. Technical issues associated with the development of an engineered osteochondral interface include the different biochemical compositions of AC and subchondral bone, as well as the different media compositions required to direct MSC differentiation into either lineage. These issues may be solved by the use of bioreactors; in our laboratory, we developed a dual-chamber perfusion bioreactor that allows the simultaneous yet separate flow of two different media to induce MSCs to differentiate specifically into chondrocytes and osteoblasts [70] (Fig. 54.1).

Although much research has focused on the treatments of osteoarthritis, a satisfactory strategy to alleviate or stop disease progression is unavailable. Preclinical studies have employed stem cells to stimulate the regeneration of the osteochondral interphase and halt the progressive destruction of the joint. These studies have used animal models that develop osteoarthritis caused by targeted genetic perturbations, surgically induced damage, or spontaneous age-associated degeneration. Stem cells can be directly injected into the defect site, usually in the early stages when the defect is limited to the cartilage only, or combined with scaffolds, in the case of exposure or degradation of subchondral bone, thereby providing mechanical support [71]. Preclinical studies for osteochondral repair have employed a handful of biphasic and triphasic scaffolds, combined with different types of differentiated or stem cells [72]. Usually small animals such as rodents and rabbits are used for proof-of-concept studies, but of course, they lack the mechanical load that can be found in humans. A few studies have employed large models, such as ovine, porcine, or equine models. The take-home message of these studies is usually a superior histological score of bone and cartilage regeneration in the experimental group compared with controls, with no sign of chronic inflammation. Stem cell–based treatment of osteochondral lesions in animal models has shown the potential to promote tissue regeneration and limit disease progression. However, osteoarthritis progression in humans is slow compared with rodents or rabbits, so the clinical use of MSCs in human is still at early stages and needs to be improved.

Tendon and Ligament

Tendons and ligaments respectively connect muscle to bone and bone to bone, producing joint motion through the transmission of contractile muscle forces and stabilizing the joint. Sharing a similar ultrastructure and biochemical content, tendons and ligaments are composed predominantly of aligned fibers of collagen type I, between which elongated fibroblasts extend in parallel. While recent studies have begun to elucidate the nascent cell populations and molecular mediators that contribute to tendon and ligament development [73], less is known about the biological events governing intrinsic healing following injury to these tissues.

Like many other musculoskeletal soft tissues, tendon and ligament possess a poor innate regenerative capacity. Rather than reconstitution of the aligned COLI fibers seen in native adult tendons and ligaments, the healing neotissue resembles a fibrovascular scar similar to that seen during wound healing, in which there is an elevated COLIII:COLI ratio, and the collagen fibrils possess a disorganized orientation [74]. Although the biochemical composition and ultrastructure remodel over years, once torn, tendons and ligaments almost never regain the structure and function they possessed before injury. Even worse, tendons and ligaments exposed to the intraarticular

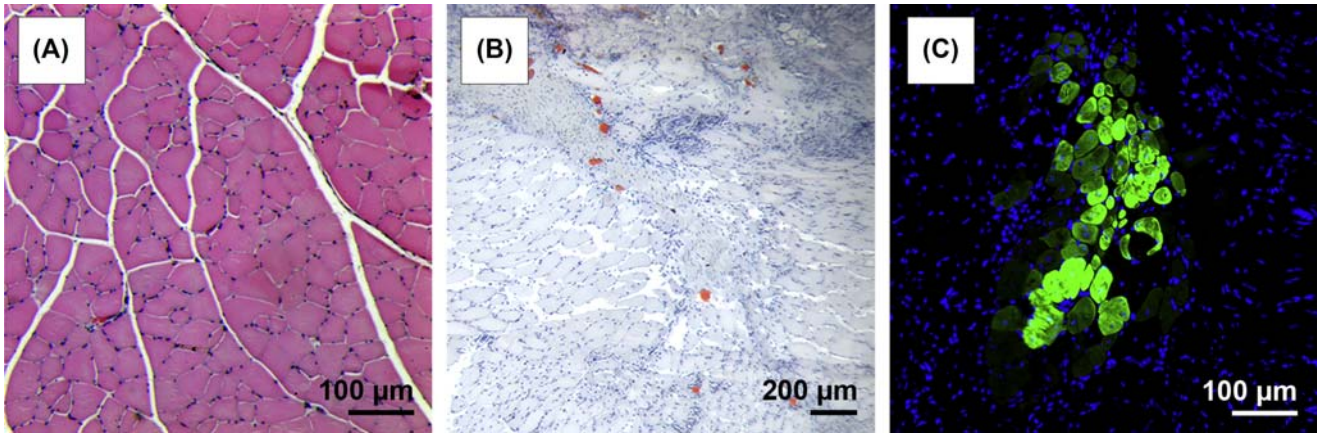

FIGURE 54.2 **Stem cell therapies for skeletal muscle diseases.** (A) Hematoxylin-eosin staining of healthy muscle in cross-section showing polygonal myofibers with multiple peripheral nuclei and minimal interstitial space between fiber bundles. (B) Oil Red O staining of degenerated muscle showing fat droplets (*red*), myofiber atrophy, and inflammatory infiltrate. (C) Green fluorescent protein muscle-derived stem cells (*green*) engrafted into the muscle of mouse model of muscular dystrophy; 4′,6-diamidino-2-phenylindole–stained nuclei (*blue*).

environment (which contains synovial fluid) often undergo negligible healing, with the resulting instability predisposing the joint to further damage, including accelerated osteoarthritis progression and damage to adjacent structures.

Because tendons and ligaments are relatively hypocellular, cell-based therapies have been explored in an effort to provide a sufficient number of reparative cells, or, as in the case of MSCs, to secrete proregenerative paracrine factors [75]. As detailed in comprehensive reviews [76,77], numerous preclinical studies have demonstrated benefit when applying stem cells to damaged tendons and ligaments. However encouraging, it must be remembered that tendon and ligament injuries are heterogeneous in character, spanning degenerative changes associated with overuse and aging (i.e., tendinopathy) to acute lacerations (i.e., flexor tendon transection). Furthermore, the tendon and ligament microenvironment, including a broad range of mechanical forces experienced in vivo, is equally diverse, from extraarticular collateral ligaments of the knee (which spontaneously heal) to the intraarticular anterior cruciate ligament (which does not heal) to the flexor tendons on the hand, which are enveloped in a sheath to which the healing tendon forms adhesions, thereby preventing normal tendon gliding. All of these details must be considered in the context of surgical or conservative treatments when developing cell-based therapies to improve tendon or ligament healing.

Such heterogeneity of injury characteristics may partly explain the paucity of studies examining the efficacy of cell-based therapies on tendon/ligament healing. A systematic review [78] identified only four studies in which stem cells were applied to damaged tendons. Two case series in which allogeneic ASCs and BM-MSCs were injected into the midsubstance of degenerated lateral epicondylar tendons [79] and patella tendons [80], respectively, showed improvements in pain and functional scores over time. However, the absence of controls limited further interpretation regarding the benefit of the stem cell injection. Similarly, Ellera Gomez et al. [81] and Hernigou et al. [82] augmented arthroscopic rotator cuff repairs with BM-MSCs injected at the repair site. Compared with the literature and/or historical controls, both studies suggested a reduction in repair failure (i.e., re-tear) and improved function. However, because those studies were also case series, Pas et al. [78] concluded that there was insufficient evidence to support the use of stem cell therapies for tendon disorders.

Tendon–Bone Interface: Enthesis

Despite a limited number of clinical studies exploring stem cell–augmented rotator cuff repair, rotator cuff tears constitute one of the greatest orthopedic challenges; over 25% of patients aged over 60 years presenting with full-thickness tendon tears [83]. Rotator cuff tears most frequently occur at the tendon–bone interface (i.e., enthesis), which normally possesses a complex architecture including a mineralized fibrocartilage gradient that serves to reduce stress concentrations in the transition from compliant tendon to stiff bone [84]. Reattaching tendon to bone is particularly challenging because the native enthesis structure is not regenerated during healing, which predisposes the surgical repair to failure and occurs at rates exceeding 90% in severe cases [85]. As a result, there has been considerable interest in applying tissue engineering principles to improve enthesis healing.

As detailed by Patel et al. [86], there have been promising tissue engineering efforts to fabricate scaffolds that mimic the gradients in cell phenotypes, ECM composition, and mineral distribution, as seen at the native enthesis. Although these emerging biomaterials may ultimately serve to guide reparative cells, whether exogenously delivered or endogenous recruited, in reconstituting the structure and function of the healing tendon−bone interface, few studies have investigated the effect of these scaffolds in vivo. On the other hand, numerous preclinical studies have been performed in which stem cells, most frequently encapsulated in a biodegradable hydrogel (e.g., fibrin, collagen), are localized between the tendon and bone, as manually apposed during surgical repair. Results have been equivocal; a review of the collective body of work suggested that stem cell therapies are most efficacious when combined with biochemical signals normally present during enthesis development (i.e., chondrogenic factors) [87]. When the molecular events underlying enthesis development are more fully elucidated, it can be expected that stem cell therapies will be modified by biochemical signals or genomic engineering to mimic the spatiotemporal expression pattern observed during the formation of this complex interface. Lessons learned in promoting enthesis healing of the rotator cuff might then by applied to other tendon−bone interfaces (e.g., ACL reconstruction), which is frequently the site of early surgical failures.

Meniscus

The menisci of the knee are crescent-shaped fibrocartilaginous structures interposed between the femur and tibia. When the knee is loaded during ambulation, the menisci serve to distribute compressive forces across the articular surfaces, thereby reducing contact stresses and facilitating joint motion. The menisci also serve as secondary stabilizers of the knee. As structure matches function, the menisci possess region-specific biochemical and ultrastructural characteristics: the inner meniscal regions, which sustain compressive forces under joint loading, are composed of an ECM rich in COLII and proteoglycans, whereas the resident cells have a round morphology and gene expression pattern similar to articular chondrocytes. Compressive loading of the inner regions creates hoop stresses that are borne by the aligned COLI fibrils of the outer meniscal regions, between which aligned fibroblastic cells are interposed. In adults, only the outer 10−30% of the meniscal width is vascularized. The hypocellularity and avascularity of the menisci, particularly the inner regions, provide a poor innate healing capacity to the meniscus when injured [88].

Unfortunately, the meniscus is the most commonly injured structure of the knee; partial removal of torn meniscal tissue (i.e., meniscectomy) constitutes the most commonly performed orthopedic surgical procedure [88,89]. Since the seminal publication of Fairbank in 1948, it has been known that meniscectomy induces the onset, and accelerates the procession, of joint degeneration (i.e., osteoarthritis) [90]. Nevertheless, the poor intrinsic healing capacity of the meniscus has limited the use of primary repairs as a treatment strategy [91]. Past efforts using blood products to enhance the healing environment, including fibrin clots [92] and trephination [93], provided inconsistent benefit, spurring the search for biological or synthetic materials that could serve as meniscal substitutes. While meniscal allograft transplantation and engineered menisci have been clinically implemented, inclusion criteria to qualify for these technologies greatly limit their widespread use [94]. Bioengineers have fabricated biological meniscal substitutes using a patient's (i.e., autologous) cells [95,96]. These technologies may ultimately offer the surgeon the option of an engineered autograft to transplant, but high fabrication costs and demanding surgical technique will likely remain challenges for the foreseeable future.

On the other hand, numerous preclinical studies have investigated the putative benefit of augmenting meniscectomies or primary repairs with stem cells, most commonly MSCs, delivered in one of two ways: (1) intraarticular injection of an MSC suspension, or (2) localized delivery to the tear site in an MSC-seeded biomaterial. For example, Hatsushika et al. [97] injected 10×10^6 allogeneic synovial MSCs suspended in phosphate-buffer saline intraarticularly 2 weeks after transection of the anterior half of the medial meniscus in a rabbit model. The regenerated meniscus in the MSC-treated group was larger than the control (no MSC) group at 1 and 3 months postinjection, but not 4 and 6 months, but it exhibited superior histological properties and chondroprotective effects at all time points. Nevertheless, the innate regenerative response of the rabbit meniscus is dissimilar to the minimal healing response seen in larger mammals, including human patients. To that end, Nakagawa et al. [98] augmented suture repairs of longitudinal tear lesions in microminipigs by intraoperatively injecting a suspension of 20×10^6 allogeneic synovial MSCs into the tear site. At 12 weeks, the MSC-treated group demonstrated improved healing, as evaluated grossly, histologically, and biomechanically, and by T1rho magnetic resonance imaging (MRI) mapping analysis. For both studies, MSCs were found at the tear site at early time points.

These preclinical studies, among others, demonstrated that MSCs could home to the meniscal lesion and improve healing. However, it has been reasoned that delivery of an MSC-seeded biomaterial to the tear site may improve

MSC localization while providing a microenvironment capable of promoting fibrocartilage formation. In support, Zellner et al. [99] reported that suture repairs augmented with MSC-seeded hyaluronan-gelatin sponges in a rabbit model enhanced fibrocartilage formation, with associated increases in biomechanical properties, compared with unrepaired defects or defects treated with acellular sponges. Similarly, Kondo et al. [100] found that aggregates of autologous synovial MSCs localized in place of the transected anterior half of the medial meniscus in aged primates promoted meniscal regeneration.

Although these studies demonstrate a clear benefit of MSCs for enhanced meniscal healing and/or regeneration, only two related clinical studies have been performed. After in vitro optimization and corroboration of benefit in an ovine model, Whitehouse et al. [101] augmented five suture repairs of meniscal tears (bucket-handle, n = 3; bucket-handle with radial extension, n = 1; and vertical flap, n = 1) with a collagen matrix (Avitene Ultrafoam collagen sponge) seeded with autologous BM-MSCs. By 15 months, two of five patients underwent meniscectomy owing to re-tear or nonhealing; the remaining three patients demonstrated MRI evidence of healing at 24 months' follow-up. Mixed results were also noted in a similar study by Vangness, Jr. et al. [102], in which intraarticular injection of 50×10^6 or 150×10^6 allogeneic BM-MSCs after subtotal meniscectomy significantly increased meniscal volume (defined a priori as a 15% threshold) in 24% and 6% of patients, respectively, as evaluated by MRI at 12 months postmeniscectomy. No patients in the control group (no MSCs) met the 15% threshold for increased meniscal volume.

Although a preponderance of preclinical studies supports the application of MSCs for improved healing, heterogeneity in study designs has prevented identification of the optimal tissue source, delivery method (i.e., intraarticular injection versus seeded biomaterial), and cell number. It also remains unclear whether allogeneic MSCs will perform worse than autologous MSCs [103]. To our knowledge, neither minimally manipulated autologous cells (derived intraoperatively, most commonly from the iliac crest bone marrow or adipose tissues) nor iPSCs have been applied in augmented meniscus repair [103].

Intervertebral Disc

The intervertebral disc (IVD) shares many structural and functional features with the meniscus. Separating each vertebrae of the spine, the fibrocartilaginous IVDs experience complex loading patterns consisting of compressive, torsional, and shear stresses. The nucleus pulposus (NP) forms a highly hydrated gelatinous core composed of COLII, proteoglycans, and chondrocyte-like NP cells, surrounded by the annulus fibrosis (AF) consisting of orthogonally oriented lamellae of aligned collagen fibers with interposed fibroblastic cells [104]. When the spine is loaded, high hydrostatic forces created in the NP are resisted by the tensile strength of the surrounding AF. In addition, IVDs possesses cartilaginous end plates on both their superior and inferior surfaces, through which oxygen and nutrients diffuse from capillaries of the subchondral bone of vertebrae. Although the outer region of the AF is vascularized, the IVD is largely avascular and hypocellular, which limits its innate healing capacity [105]. In the context of a complex interplay of genetic and environmental factors, this feature is thought to predispose the IVD to degenerative changes beginning as early as the second decade [106].

IVD degeneration is one of the principal contributors to low back pain, which is the leading cause of disability in the developed world and is estimated to affect 75–80% of people at some stage [107]. Although the molecular mechanisms governing IVD degeneration are incompletely understood, early loss of proteoglycan content from the NP is associated with decreased disc height, concurrent with weakening of the AF in the context of increased catabolic and inflammatory mediators. Upregulated inflammation, coupled with aberrant neovascularization and innervation of the IVD, is thought to contribute to discogenic pain and deterioration of spine mobility. Furthermore, the weakened AF can eccentrically bulge or fail, permitting herniation of NP contents, causing radiculopathy pain by aggravation of adjacent nerve roots. At present, there are no efficacious treatment options between conservative measures (i.e., physical therapy, nonsteroidal antiinflammatory drugs, epidural corticosteroid injection) and surgical intervention (i.e., microdiscectomy, total discectomy, disc arthroplasty, spinal fusion) [104]. Therefore, regenerative and tissue engineering strategies have been explored as a means of reversing or preventing further disc degeneration.

In a comprehensive review of cell-based therapies for lumbar degenerative disc disease, Oehme et al. [107] identified 25 preclinical studies in which stem or progenitor cells were applied to promote IVD regeneration. Although 20 of 25 studies investigated MSCs, the cell phenotype was variable (i.e., autologous versus allogeneic versus xenogeneic, bone marrow- versus adipose-derived), as was the intradiscal delivery method (i.e., suspension versus hydrogel). Furthermore, the animal models included species from rats to sheep, yet most involved nucleotomy or NP aspiration from the disc of a previously young, healthy animal. Compared with untreated controls, MSC-treated

groups generally enhanced disc regeneration, as demonstrated by increased disc height, improved ECM synthesis, and superior morphology, as evaluated by MRI [107]. Where degenerative disc disease has progressed beyond any reasonable hope of cell-augmented repair, disc replacement with a tissue-engineered graft may ultimately be possible. As highlighted by Bowles and Setton [108], composite strategies for tissue engineered IVD replacements have largely used biomaterials consisting of fibrous polyesters (e.g., PLA) for the AF and MSC-seeded hydrogels for the NP. Preclinical results have been encouraging, although limited in the number and length of follow-up.

Only four clinical studies have explored cell-based treatment of IVD degeneration. In the earliest report, Yoshikawa et al. [109] implanted collagen sponges seeded with autologous MSCs (isolated from iliac crest bone marrow 2–4 weeks earlier) into a single degenerative disc in two patients. Both patients experienced improvement in pain and diminished radicular symptoms by 6 months, which persisted to the final follow-up at 2 years. T2-weighted MRI at 2 years suggested increased water content compared with preoperative MRI. Similar findings were subsequently reported in cases series by Orozco et al. [110] and Pettine et al. [111], in which degenerated IVDs were injected with suspensions of BM-MSCs or bone marrow concentrate, respectively. Using a more extensive coculture protocol, Mochida et al. [112] injected autologous NP cells culture expanded in direct contact (i.e., cell-to-cell) with autologous BM-MSCs into degenerative IVDs immediately adjacent to a spinal fusion (the discarded disc rendering the autologous NP cells). Compared with the studies employing MSCs, only one of nine patients demonstrated mild improvement in disc morphology as evaluated by MRI. That said, experimental design varied substantially across studies, limiting rigorous comparison. Furthermore, none of these clinical studies included a control patient population.

Skeletal Muscle

Despite remarkable regenerative ability, skeletal muscle integrity and function are often compromised in muscular dystrophy [113–115]. Muscular dystrophy describes a heterogeneous group of approximately 40 inherited disorders characterized by progressive muscle weakness, degeneration, and wasting. Duchenne muscular dystrophy (DMD) is one of the most common and severe childhood muscular dystrophies; however, there not yet effective treatments available for DMD patients [116]. In addition to diseases, loss of skeletal muscle through trauma, tumor ablation, and prolonged denervation represent common clinical challenges. To treat traumatic muscle loss, free tissue transfer is an option, but autologous muscle transfer not only causes donor site morbidity, it can produce loss of function at the donor site [117]. Tissue engineering of skeletal muscle to replace functional muscle tissue could offer an alternative.

Engineering functional skeletal muscle would require the recapitulation of functional motion and integration with host connective tissues [118]. At the cellular level, it is also important to achieve parallel alignment of fibers and integration of functional neuromuscular junctions. As with other musculoskeletal tissues, mechanical stimulation is essential during myogenesis; it influences metabolic activity and gene expression, as well as fiber alignment. Also, myoblasts, which are similar to many types of cells, lose their differentiation capacity as they are culture-expanded [119,120]. Therefore, understanding the biology of precursor cell types such as SCs and other muscle progenitor cells, is crucial. Although SCs act as local regenerative cells in muscle, their limited expansion potential in vitro limits their current usefulness relative to other stem cell sources [121]. Such stem cells include, but are not limited to, ESCs, iPSCs, BM-MSCs, ASCs, hematopoietic stem cells (HSCs), and muscle-derived stem cells (MDSCs) [120,122,123].

ESCs and iPSCs, which are pluripotent cells (PCs), are considered the most promising cell source for cell-based therapies. Studies have reported that these cells can be induced and successfully converted into skeletal muscle cells to repair acutely and chronically injured muscle [120,122]. Pyle and colleagues reported that iPSCs derived from DMD patients' skin fibroblasts were successfully reprogrammed to muscle progenitor cells. Upon transplantation of these human iPSCs, they underwent efficient fusion and improved ultrastructural organization and the expression of adult myosin in mice [124]. However, concerns regarding tumorigenic potentials and complex biological and ethical issues correlated with PCs have dampened their use in favor of stem cell therapies.

Most research thus far has focused on MSCs from bone marrow, fat, and blood. Starting with the study of Ferrari et al. in 1998, which involved the transplantation of BM-MSCs [125], several studies have reported that transplantable ASCs also could be recruited to injured muscle and take part in repair and regeneration [123]. Transplantation of HSCs has shown to result in increased dystrophin gene expression in dogs with DMD [126]. In addition to their role in differentiating into myofibers, MSCs have been shown to impose paracrine effects on differentiation and tissue regeneration of host resident muscle progenitor cells [120,127].

Besides MSCs, MDSCs show promise for stem cell–based therapies for the treatment of DMD (Fig. 54.2) [128]. MDSCs are isolated from late-plating mononucleated muscle cells and are long-term self-renewing cells [129]. It was previously reported that compared with myoblasts, the MDSCs demonstrated a higher intramuscular engraftment capability in vivo [130,131]. The exact mechanism involved in the improved regenerative potential of the MDSCs remains unclear; however, several studies suggested that their high survival property owing to elevated resistance to stress is critical, as well as their release of soluble factors such as VEGF [132,133] (Fig. 54.2).

In vivo, the ECM of muscle provides fibers with the architecture to support development and function. Therefore, for skeletal muscle tissue engineering, a scaffold may be needed to mimic the matrix and maximize the potential therapeutic effects of cells [134]. A suitable biomaterial should be used to fabricate the scaffold to guide proper tissue reorganization, provide optimum microenvironmental conditions for cells, and coordinate in situ tissue regeneration. The most commonly used materials for scaffold preparation are collagen, naturally derived materials, and embedded 3D collagen with muscle stem cells, which has been shown to enhance cell attachment, expansion, and differentiation. Most synthetic scaffolds used for skeletal muscle tissue engineering are fabricated from polyesters such as PGA and polylactic glycolic acid, which can easily be tailored into shape- and size-specific scaffolds with easy control over mechanical, structural, and physicochemical properties. Injectable, cell-compatible hydrogel is often used as a cell carrier because of its capacity to promote myogenic cell differentiation in vivo [118,135]. The presence of growth factors, including VEGF, insulin-like growth factor-1, hepatocyte growth factor, stromal cell-derived factor-1, and FGF, is also an important element of muscle engineering, because these growth factors can accelerate muscle stem cell activation, proliferation, and differentiation [136].

Although clinical trials on muscle tissue engineering of human subjects are limited, several clinical trials in patients with muscular dystrophy involving these stem cells have progressed (ClinicalTrials.gov). Table 54.1 summarizes clinical trials from 2004 to 2019.

CHALLENGES AND PROSPECTS

Now some time after the seminal publications of Langer and Vacanti [137] and Brittberg et al. [24], respectively, popularizing the concept of tissue engineering and describing the application of autologous chondrocytes for AC regeneration, the clinical impact of stem cell therapies for musculoskeletal diseases remains limited. Although numerous in vitro and preclinical studies demonstrated the potential of stem cell therapies, as highlighted previously, there are numerous challenges preventing clinical translation, including obvious outstanding technological and biological questions, but also regulatory and financial considerations [138].

An alternative approach to current cell-based therapies involving two-step surgical procedures with intervening ex vivo cell expansion is to bolster the reparative functions of endogenous stem cells. Because nearly all tissues contain endogenous stem cells that contribute to normal homeostatic function, it is hypothesized that directed modulation of the stem cell niche may overcome the microenvironmental cues in injury or degeneration that otherwise inhibit complete regeneration [139]. Furthermore, the immune response to disease or damage is increasingly recognized as a principal determinant of subsequent healing; targeted modulation of the immune response by novel biomaterials and/or biologics and pharmaceuticals may promote synergism with stem cell therapies, thereby improving stem cell viability and synthetic function [140]. Along the same vein, emerging biomaterials localized at the musculoskeletal lesion have been shown to be capable of recruiting endogenous stem cells to the scaffold where embedded bioactive growth factors can drive site-appropriate differentiation [141].

Exogenous stem cell therapies may still prove valuable, either to reconstitute damaged tissues directly or to enhance healing indirectly by secreting regenerative paracrine factors. Because MSCs have been the most commonly used cell phenotype for cell-based therapies, better characterization of their phenotype and mechanism of action will be required to maximize their benefit [142]. To that end, it remains an open question whether allogeneic MSCs induce an adverse inflammatory response [143]. Although limited in number and scope, studies suggest that matching of major histocompatibility complex (MHC) between donor and recipient patients may be necessary. If MHC-matched allogeneic MSCs indeed prove equivalent in efficacy to autologous MSCs, they would represent an off-the-shelf source for cell-based therapies, obviating the current need for two-step procedures and ex vivo cell expansion.

Alternatively, but not mutually exclusive, it may be possible through emerging genome editing technologies to silence otherwise immunogenic elements of donors cells. At the same time, these technologies may allow for the creation of autonomously regulated, closed-loop delivery of biological drugs. For instance, using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated-9 system, Brunger et al. [144]

TABLE 54.1 Clinical Trials of Cell Therapies in Muscular Dystrophies From 2004 to 2019

ClinicalTrials.gov Identifier	Number of Patients	Muscular Dystrophy	Cell Therapy	Patient Age	Study Start and End Dates	Effects
NCT00773227 Phase 2	30	OPMD	Autologous myoblast	18–75	Apr. 2004–Oct. 2015 [151]	Improvement in video endoscopy and video fluoroscopy of swallowing
NCT02241434 Phase 1	245	DMD	Autologous BM mononuclear cells	3–25	Jan. 2009–Jun. 2016	No report
NCT02285673 Phase 1/2	10	DMD	Umbilical cord mesenchymal stem cells	7–20	Nov. 2013–Nov. 2015	Primary outcome will be assessed by DMD gene expression, no report
NCT01834040 Phase 1/2	30	DMD	BM-derived autologous cells	4–30	Sep. 2014–Oct. 2016	Primary: Improvement of daily living scale Secondary: improvement of muscular dystrophy specific functional rating scale
NCT01834066 Phase 1/2	25	DMD	BM-derived autologous cells	6–25	Sep. 2014–Dec. 2016	No report
NCT02208713 Phase 1	21	FSHD	MDSCs and ASCs	18–50	May 2014–Dec. 2017	No report
NCT02196467 Phase 1/2	10	DMD	Myoblast	16+	May 2014–Feb. 2019	No report

ASCs, adipose derived stem cells; *BM*, bone marrow–derived; *DMD*, Duchenne muscular dystrophy; *FSHD*, Facioscapulohumeral muscular dystrophy; *MDSCs*, muscle-derived stem cells; *OPMD*, oculopharyngeal muscular dystrophy.

program iPSCs to respond to proinflammatory cytokines interleukin-1 and tumor necrosis factor α (TNF-α) by synthesizing and secreting their soluble inhibitors (e.g., soluble TNF receptor 1). By engineering these feedback loops into the iPSC genome, the soluble inhibitor was synthesized only when cultured in the presence of its respective cytokine. Although the concern for tumorigenicity for iPSCs is real, it is possible to differentiate the iPSC into an MSC (or further into a specific cell phenotype, e.g., chondrocyte) [145], thereby mitigating the risk of teratoma formation upon in vivo application. If proven safe and effective, iPSCs, with or without further genetic engineering, might provide the ultimate off-the-shelf source for cell therapies, including diseases of every organ system.

Successful translation of cell therapies to the clinic would also be facilitated by improved model systems, both animal and in vitro, which reflect the clinical scenario and human physiology more accurately. Whereas many musculoskeletal diseases amenable to stem cell therapies are chronic conditions in aged patients, most animal models have used young, healthy animals in which an iatrogenic damage is acutely induced in a tissue, immediately followed by the experimental intervention. Fortunately, the discrepancy between established animal models and human patients is increasingly recognized, with ongoing development of models of chronic diseases in aged animals. Alternatively, companion animals (i.e., pets) often experience naturally occurring diseases and injuries similar to those of humans, which offers opportunities to investigate stem cell-based therapies in a model of greater clinical relevance [146]. That said, no animal model perfectly matches human disease; drug therapies developed through extensive animal testing ultimately prove ineffective or even harmful when translated into clinical therapies. As a result, there has been an increased emphasis on developing microphysiological in vitro models (i.e., organs-on-a-chip) using only human cells [70,147]. It is hypothesized that human-specific microphysiological models will allow greater fidelity to human pathology while also obviating the challenges, including biological, financial, and ethical considerations, of animal models. First-of-kind models of IVDs [148] and osteochondral interfaces of joints [149] have shown promise; the latter were discussed earlier.

When stem cell therapies, whether optimized in microphysiological or animal models, are translated into human patients, higher-quality clinical studies are needed to validate their efficacy. Most clinical trials of stem cell therapies for musculoskeletal diseases are case series that do not include adequate controls. In addition,

evaluation of stem cell–mediated enhancement of tissue regeneration would be facilitated by novel noninvasive metrics of tissue quality, whether biochemical or imaging-based [150]. These technologies would allow real-time monitoring of tissue healing, compared with histological evaluation after death in an animal model. In turn, this would facilitate the researcher's ability to investigate stem cell therapies in human patients.

List Abbreviations and Acronyms

AF Annulus fibrosis
ASC Adipose-derived stem cell
BM-MSC Bone marrow–derived mesenchymal stem cell
DMD Duchenne muscular dystrophy
ECM Extracellular matrix
ESC Embryonic stem cell
HAp Hydroxyapatite
HSC Hematopoietic stem cell
hMSC Human mesenchymal stem cell
FGF Fibroblast growth factor
iPSC Induced pluripotent stem cell
IVD Intervertebral disc
MDSC Muscle-derived stem cell
MHC Major histocompatibility complex
MSC Mesenchymal stem cell
NP Nucleus pulposus
PGA Polyglycolic acid
PLA Poly-lactic acid
SC Satellite cell
TNF-α Tumor necrosis factor-α
VEGF Vascular endothelial growth factor

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CHAPTER

55

Myoblast Transplantation in Skeletal Muscles

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INTRODUCTION

Cell transplantation, which is frequently called "cell therapy," is an approach under study to treat different skeletal muscle disorders. The starting point of this therapeutic approach can be traced to 1978, when Partridge and colleagues proposed that "in subjects suffering from inherited recessive myopathies, muscle function might be restored if normal myoblasts could be made to fuse with defective muscle fibers" [1]. Among these myopathies, Duchenne muscular dystrophy (DMD) was the first target and has being the main objective of this experimental therapeutic strategy. This is because of the relative frequency of this disease (a prevalence of 50 cases per million in the male population) and its severity: progressive skeletal-muscle degeneration in the limbs and trunk during childhood and adolescence, leading to motion loss, respiratory insufficiency, and ultimately death by respiratory or cardiac complications. Other clinical applications subsequently sought the treatment of individual muscles affected by different processes, such as the cricopharyngeal muscle in patients with oculopharyngeal muscular dystrophy [2], the tibialis anterior in patients with fascioscapular muscular dystrophy [3], the urinary sphincter in patients with stress urinary incontinence [4], and the external anal sphincter in patients with anal incontinence [5].

The saga of cell transplantation in clinical myology is an example of the importance of the preclinical basis for designing new therapeutic protocols. After a few animal experiments in the 1980s, mostly in mice, several groups undertook clinical trials of cell transplantation in the 1990s [6]. Lacking adequate preclinical data to plan the strategies of cell implantation and even the strategies to control acute rejection, these clinical trials reported scarce and modest results on the molecular level. In fact, these trials were conducted in the hope that some muscle precursor cells injected in a few sites of a skeletal muscle would be able to diffuse throughout the muscle and spontaneously fuse with so many myofibers that a therapeutic effect would be obtained. Subsequent research demonstrated that this hope was unrealistic, and the lesson to take from this experience is that clinical researchers need to know the behavior of the grafted cells in appropriate experimental conditions to design efficient clinical protocols.

This chapter addresses current knowledge that could be useful for future clinical applications of cell transplantation to treat skeletal muscle pathologies. For this reason, and considering warnings regarding the poor clinical predictability of studies in mice [7,8], priority will be given to observations made in humans and nonhuman primates. The chapter is organized to address three main questions: (1) why myoblasts meet the properties needed for the aim of the treatment, (2) how the muscle precursor cells can be properly delivered to the target tissues, and (3) how to ensure the long-term survival of the graft.

SATELLITE CELL–DERIVED MYOBLASTS MEET THE PROPERTIES NEEDED FOR TRANSPLANTATION IN SKELETAL MUSCLES

The term "myoblast" defines the proliferating mononuclear progenitor cells that fuse with analogous cells to form myotubes, the syncytial stage in the process of myogenesis that, under adequate conditions, precedes myofiber formation. This applies to every case in which this phenomenon occurs; that is, during skeletal muscle embryogenesis, in postnatal myofiber regeneration, and in vitro culture of skeletal muscle cells. To be specific in the terminology, avoiding confusion between myoblasts involved in postnatal muscle regeneration or proliferated in vitro from

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skeletal muscle biopsies (both derived from satellite cells) and somite-derived myoblasts involved in muscle embryogenesis, we will use the term "satellite cell–derived myoblasts" (SCDMs) for the first two cases.

Any cell transplantation strategy involves the graft of either differentiated cells or precursor cells with the ability to differentiate into the former. In the skeletal muscle, the differentiated cells responsible for muscle contraction, that is, the myofibers, are not proper for transplantation in clinical regenerative medicine because they are long multinucleated syncytia that cannot be proliferated in vitro or adequately implanted to rebuild a muscle. The possibility of a more efficient cell transplantation in this context is offered by the specific stem cell of the skeletal muscle: that is, the satellite cell. The main function of satellite cells is to repair myofiber damage. They are normally quiescent in the periphery of myofibers until an injury produces focal or total necrosis of a nearby myofiber, triggering a process that involves the removal of myofiber debris by phagocytic cells and the activation of dormant satellite cells, which differentiate into proliferating SCDMs. Murine models demonstrate that muscle regeneration is not possible when satellite cells are depleted [9–11], which confirms that the mainstay of muscle regeneration is the sequence: satellite cells → SCDMs → myotubes → myofibers. Satellite cells can be isolated from skeletal muscle biopsies by standard cell-culture techniques and can be expanded as SCDMs in vitro, maintaining their capacity to fuse into myotubes that will differentiate into myofibers under appropriate conditions. This relative simplicity to obtain and proliferate these cells in vitro facilitated the use of SCDMs for cell transplantation.

Relevant Properties of Satellite Cell–Derived Myoblasts for Cell Transplantation in the Skeletal Muscle

Lipton and Schultz reported for the first time in 1979 the two main myogenic properties of exogenous SCDMs implanted into skeletal muscles [12]: (1) they fuse with the recipient's myofibers, and (2) they form new small myofibers by fusing the grafted cells among themselves. The first property allows gene complementation, that is, myofibers in which exogenous myogenic cells are incorporated express proteins coded by the exogenous and endogenous myonuclei [13], which are thus referred to as "mosaic" or "hybrid" [14]. Through gene complementation, grafted SCDMs can act as vehicles of therapeutic genes, for example by introducing normal genomes in the genetically abnormal myofiber of a patient with a recessive genetic myopathy. The second property opens the door to the possibility of forming new myofibers in patients in which the skeletal-muscle parenchyma has been lost. A third property of SCDM transplantation, which was reported later, is the possibility of developing into new graft-derived satellite cells. Fig. 55.1 illustrates the properties, in terms of myogenic outcomes, of SCDM transplantation.

Gene Complementation

The first experimental demonstration of this phenomenon as a consequence of cell transplantation in the skeletal muscle was reported by Partridge et al. in 1989 [15]. They transplanted normal mouse SCDMs in *mdx* mice (which, as patients with DMD, have a myopathy caused by the deficiency of the protein named dystrophin) and observed several myofibers expressing dystrophin a few weeks later. The same observation was repeated soon by other researchers [16,17] and is now routine in research in this area. Other proteins restored by normal SCDM transplantation in mouse models of muscular dystrophies were merosin in *dy/dy* mice (a model of merosin-deficient congenital muscle dystrophy) [18] and dysferlin in SJL mice (model of limb-girdle muscular dystrophy 2B) [19]. In humans, occasional observations of increased dystrophin expression after allotransplantation in DMD patients of SCDMs from nonmyopathic donors were reported during clinical trials conducted in the 1990s, but these observations were not conclusive and most patients gave negative results [6]. It was not until more recent clinical trials, based on translational preclinical studies in nonhuman primates, that it was shown that donor-derived dystrophin can be expressed systematically in the muscles of patients with DMD locally injected with normal SCDMs (Fig. 55.2) [20–23], provided certain conditions are met (as explained subsequently).

A critical factor complicating the protocol of intramuscular cell implantation (in the cases in which we sought to obtain a homogeneous and significant genetic complementation in a whole large muscle) is that the intracellular proteins encoded by a single myonucleus remain localized near the nucleus of origin, in a region named the "nuclear domain" [24]. This restriction is produced by the limited diffusion of both the messenger RNA (mRNA) [25] and protein [26]. Consequently, proteins from graft-derived myonuclei are expressed only in the myofiber segments where fusion of the grafted cells was produced (Fig. 55.1E). The size of the nuclear domain depends on the capacity of a given protein to diffuse or remain anchored to stationary cellular components [26]. As an example, single injections of ß-galactosidase–labeled normal SCDMs into *mdx* mice produced dystrophin expression throughout

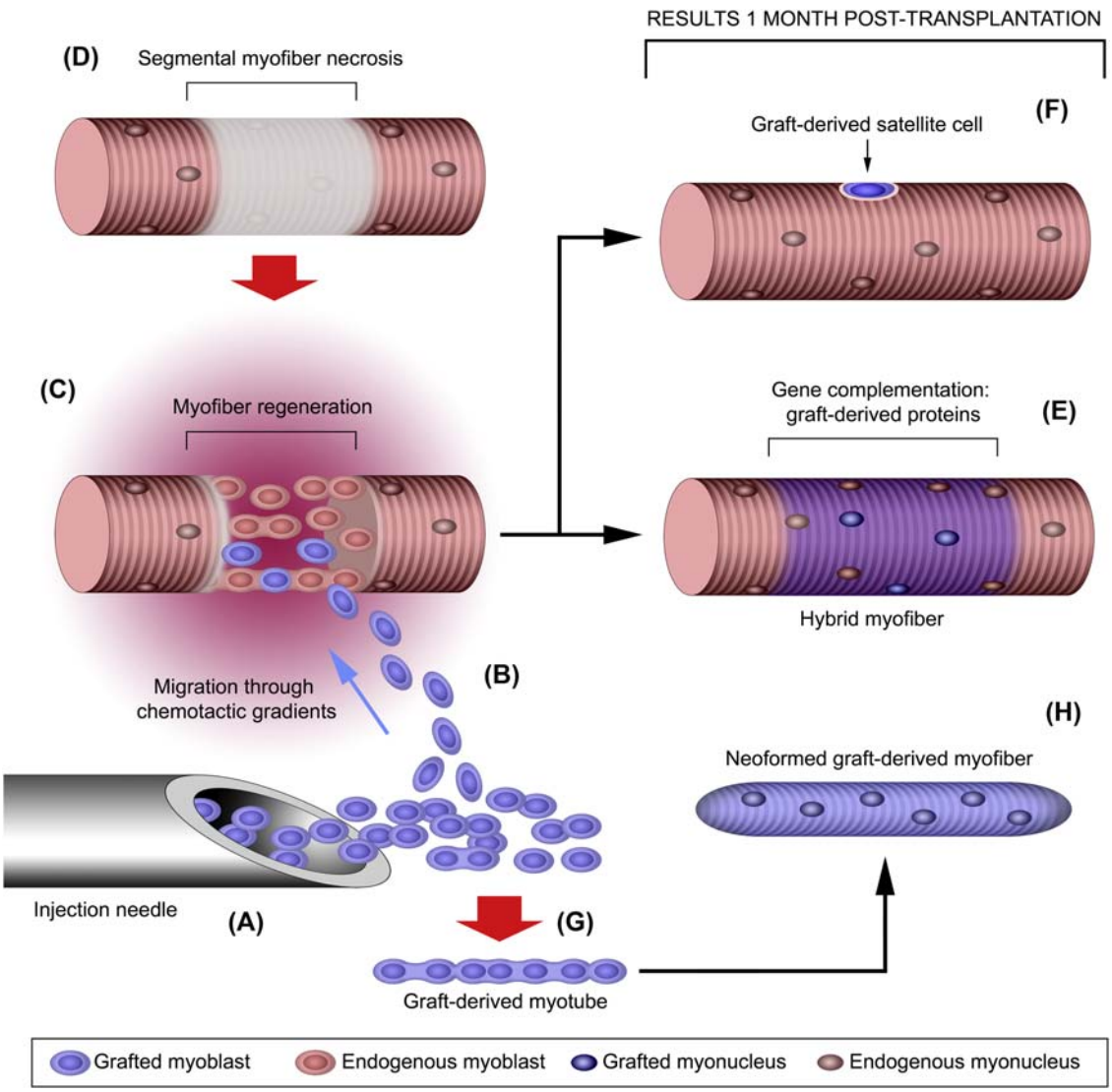

FIGURE 55.1 Myogenic properties of satellite cell–derived myoblasts (SCDMs) after injection in a skeletal muscle. Grafted elements are represented in *blue* and endogenous in *red*. After their intramuscular injection (A), some SCDMs migrate (B) through chemotactic gradients (*pink gradation*) toward regions of myofiber regeneration (C), which is the consequence of myofiber damage leading to segmental necrosis (D). Grafted SCDMs are recruited in this myofiber regeneration, fusing with the endogenous SCDMs (C). When regeneration is completed, the coexistence of grafted myonuclei with endogenous myonuclei leads to a hybrid myofiber (E), in which genetic complementation leads to the expression of graft-derived proteins around the graft-derived myonuclei. Some grafted cells, on the other hand, can develop into new graft-derived satellite cells (F). Finally, some grafted SCDMs can fuse among themselves to form myotubes (G) that under adequate conditions become new graft-derived myofibers (H).

roughly 500 μm of the myofiber, in contrast with 1500 μm for β-galactosidase [27]. This difference was even more striking when dystrophin expression was compared with green fluorescent protein [28]. The longer domain of β-galactosidase and green fluorescent protein should be attributed to the solubility of these proteins, resulting in spreading more than dystrophin, which remains attached to the cytoskeleton.

Formation of New Myofibers

In DMD and other degenerative myopathies, progressive worsening of muscle weakness is produced by the steady and irreversible loss of myofibers. An ideal treatment for the advanced stages of these diseases may include not only molecular correction but also restoration of functional myofibers.

The potential of SCDM transplantation to restore skeletal muscle mass and strength was reported in mice after acute severe muscle destruction [29–31]. These experimental conditions, however, are not similar to those of a

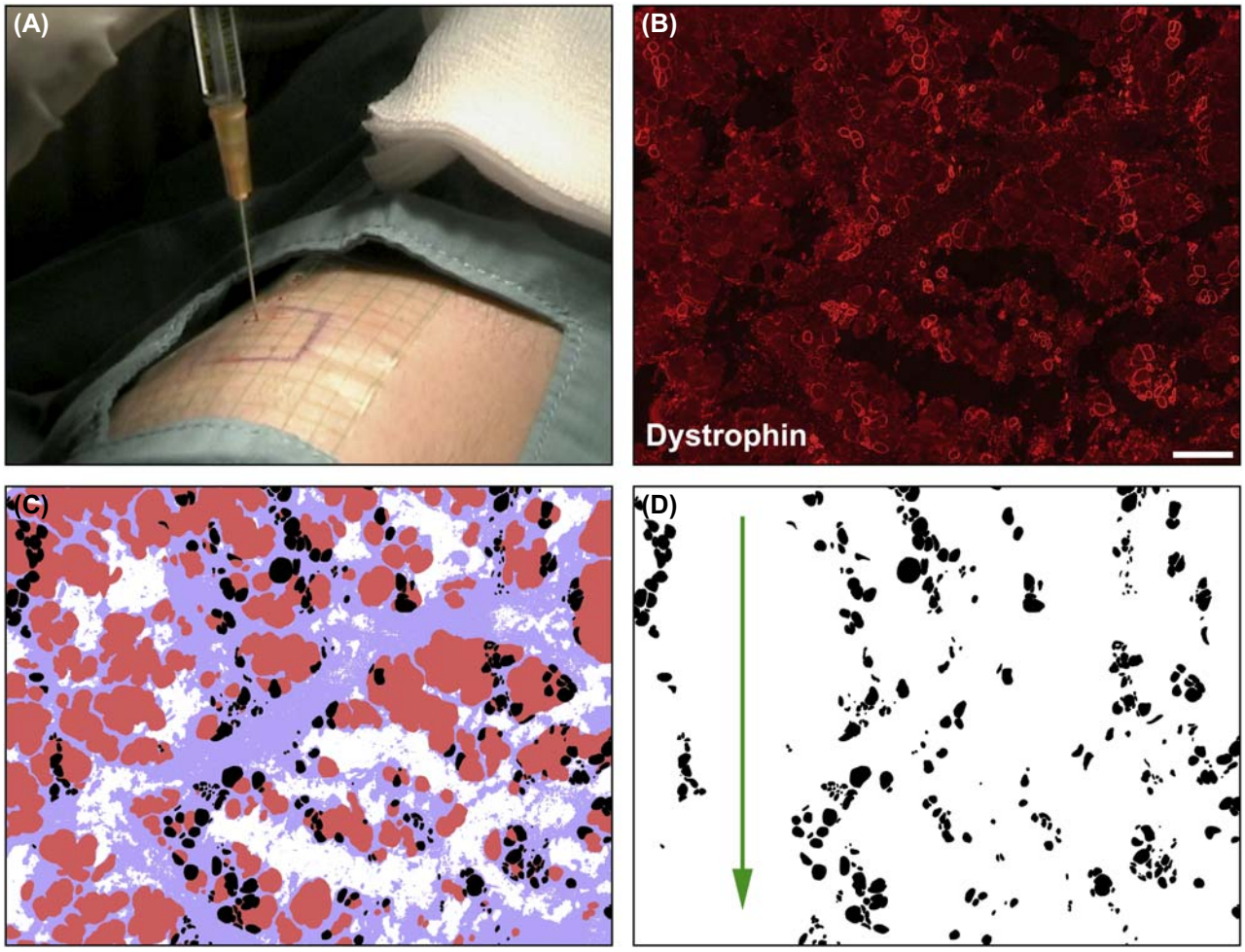

FIGURE 55.2 Allotransplantation of normal satellite cell–derived myoblasts in 1 cm^3 of the tibialis anterior of a patient with Duchenne muscular dystrophy, in a Phase 1A clinical trial conducted by the authors. (A) Several parallel intramuscular cell injections are being done with a 100-μL Hamilton syringe. The cells are delivered homogeneously during the needle withdrawal, and the density of cell injections is controlled with the help of a sterile transparent dressing with a grid. (B) The cross-section of a biopsy performed 1 month later in a cell-grafted site is shown stained for the red fluorescent immunodetection of dystrophin. (C) The schematic representation of the histological image in (B) illustrates the distribution of the dystrophin-positive myofibers in this biopsy: in *black*, dystrophin-positive myofibers; in *red*, muscle tissue; in *blue*, connective tissue; the *white spaces* correspond essentially to fat tissue. (D) Isolating the dystrophin-positive myofiber profiles of the previous image, it can be seen that they are distributed roughly in parallel axes corresponding with the original trajectories of the injections (indicated by the *arrow*). (B) Scale bar = 500 μm.

degenerative myopathy in which the damage is progressive and leads to a loss of the tissue scaffold owing to the fibrotic and fat replacement [6]. The tissue scaffold, which remains preserved in the acute muscle damage experiments in mice but is lost in degenerative myopathies, seems essential for the regeneration of myofibers [32]. How cell transplantation might form new functional myofibers in skeletal muscles that have degenerated to fibrosis and/or fat substitution remains insufficiently studied. A study in mice suggested that it could be possible to create myotubes within the adipose tissue [33]. Otherwise, neoformation of small ectopic muscles was observed after subcutaneous implantation of SCDMs in mice, despite the absence of a previous endomysial support [34]. In *mdx* mice, formation of new myofibers through the fusion of the implanted SCDMs among themselves was observed after irradiation of the recipient muscle [35].

Progressing from these few observations to a clinically functional procedure remains a challenge, among other factors, because these results were obtained in mice, which have intrinsically greater muscle regeneration capacity than do primates [36]. A clinical observation encouraging this research is the presence of small dystrophin-positive myofibers, putatively neoformed, in patients with DMD who were transplanted with normal SCDMs (Fig. 55.3) [20].

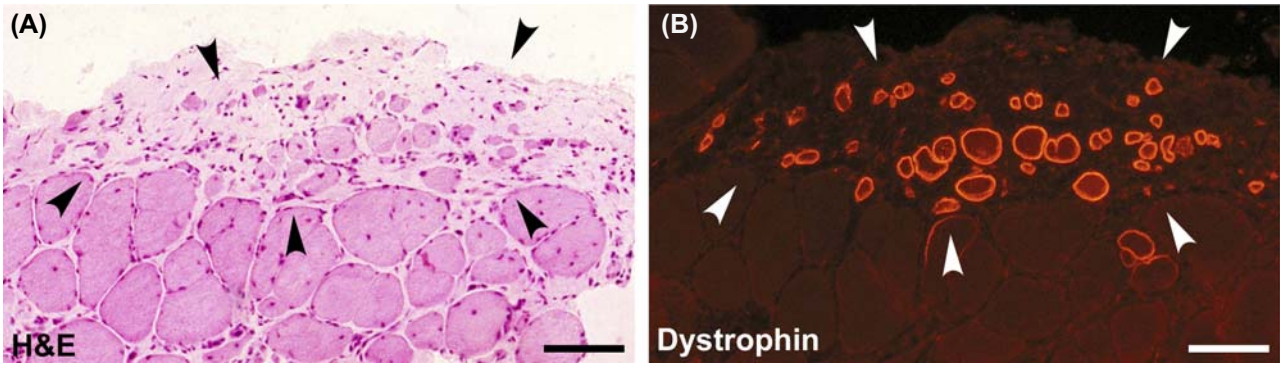

FIGURE 55.3 Probable neoformation of small graft-derived myofibers after satellite cell–derived myoblast (SCDM) transplantation in a patient with Duchenne muscular dystrophy (DMD). The figure shows serial cross-sections of a muscle biopsy performed in the tibialis anterior of a 16-year-old patient with DMD, transplanted 1 month before with SCDMs proliferated in vitro from a muscle biopsy from his father. Sections were stained with hematoxylin-eosin (H&E) (A) and for fluorescent immunodetection of dystrophin with MANEX50, an antibody that detects an epitope coded by exon 50 of the dystrophin gene (B). Because this was the exon deleted in this patient, this antibody confirms that this dystrophin is of donor origin. A cluster of small myofibers dispersed into an extrafascicular accumulation of connective tissue is indicated between *arrowheads*. All of these small myofibers exhibit donor-derived dystrophin. Studies in nonhuman primates supported the interpretation that this corresponds to myofiber neoformation caused by the fusion of the grafted SCDMs among them in the connective tissue. Scale bars = 100 μm.

Formation of Graft-Derived Satellite Cells

Transplants of mouse and human SCDMs into mouse muscles showed that some grafted SCDMs remained as mononuclear cells able to participate later in muscle regeneration and also proliferate and form myotubes in vitro [37–39]. Morphological studies showed specifically that graft-derived satellite cells were produced by the transplantation of mouse SCDMs [31,34,40] and human SCDMs [41,42] in mouse muscles (Fig. 55.4). Some observations suggest that this phenomenon could also occur after SCDM transplantation in humans: Donor-derived mononuclear cells were detected in the muscles of patients with DMD who received SCDM allotransplantations from nonmyopathic donors, and some of the donor-derived nuclei were in locations susceptible to corresponding to satellite cells [20,43].

This may imply that the potential therapeutic effect of SCDM transplantation is not limited to the early fusion of the implanted cells, but should also ensure a permanent source of normal satellite cells to participate in muscle hypertrophy and regeneration. Moreover, this could mean that the percentage of myofibers expressing donor-derived dystrophin may increase over time, if a process similar to that described in *mdx* mice regarding the expansion of clusters of myofibers expressing revertant dystrophin is produced [44].

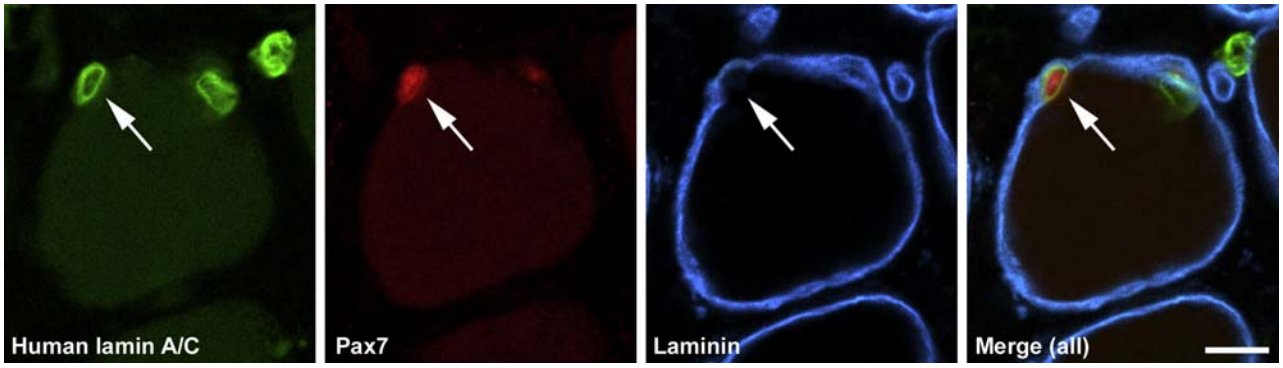

FIGURE 55.4 Graft-derived satellite cells formed by the transplantation of human satellite cell–derived myoblasts (SCDMs) in immunodeficient mice. The figure shows a single myofiber in a cross-section of a skeletal muscle of an immunodeficient mouse transplanted 4 weeks before with SCDMs from an adult human. Different elements are evidenced by fluorescent immunodetection in confocal microscopy. The peripheral nuclear labeling of human lamin A/C (*green fluorescence*) evidences two human nuclei in the periphery of the myofiber (visible by contrast enhancement). One of them (*arrow*) shows a typical intranuclear staining of Pax7, a marker of satellite cells (*red fluorescence*). Immunodetection of laminin (*blue fluorescence*) evidences that this nucleus is in the periphery of the myofiber inside the basal lamina: that is, in the anatomical position of a satellite cell. Scale bar = 10 μm.

CELL ADMINISTRATION

Once appropriate cells for transplantation are produced, the following step is to deliver them to the target tissue in such a way that the cell graft will reach a therapeutic objective. Two main routes have been explored for cell transplantation in myology: local intramuscular injection and intravascular infusion.

Intravascular administration, essentially by the intraarterial route, was reported to produce significant levels of gene complementation using some cell types under specific experimental conditions in mice and dogs [45–48]. Among these cells, the so-called "mesoangioblasts" were considered the most attractive for cell therapy in myology, given the excellent results of gene complementation reported in mice and dogs by one group [45,46]. In contrast, a clinical trial of intraarterial administration of mesoangioblasts in patients with DMD gave negative results [49], questioning the clinical predictability of the previous animal studies. With regard to SCDMs, few studies of intraarterial infusion have been performed in rats [50] and nonhuman primates [51]. In both cases, it was observed that the SCDMs were able to fuse with myofibers, depending on a concomitant mechanical damage in the muscles [50,51]. The analysis of nonhuman primate tissues showed that the intraarterially injected SCDMs were mostly trapped in the capillary network of the limb irrigated by the injected artery (in all tissues analyzed) and that the production of microemboli in the arterioles produced small muscular microinfarcts [51].

Because the only positive results confirmed in humans have been obtained with local administration of SCDMs by intramuscular injections, the rest of the chapter will be dedicated to this route of administration.

Density of Cell Injections

The main constraint of the intramuscular route is that the injected cells contribute to muscle regeneration essentially around the injection trajectories, and mostly in the myofibers damaged during the injection. This is clearly observed in nonhuman primates, in which each SCDM injection leads an irregular "strip" of hybrid myofiber profiles in histological muscle cross-sections [52–55], constituting a histological pattern called "bands of engraftment" [55] (Figs. 55.5 and 55.6). An almost similar pattern, although sometimes less defined, was observed after injections of normal SCDMs in patients with DMD (Fig. 55.2) [20,22].

Because protein expression is limited to nuclear domains, which are short in the case of dystrophin, as mentioned earlier, cell injections must be placed close to each other and must reach the whole muscle to obtain a homogeneous expression of graft-derived proteins throughout a muscle in which the only myofiber regeneration that occurs is produced by the injection damage. Consequently, the extent of gene complementation in a muscle will depend on the density of the matrix of cell injections: that is, on the number of injections per surface of the matrix (Fig. 55.5C and D) [53]. The highest percentages of dystrophin-positive myofibers after normal SCDM transplants in DMD patients were indeed obtained when at least 100 cell injections/cm^2 were performed [20–23]. This form of cell implantation was denominated "high-density injections" [6], to make a distinction from the earlier clinical trials of SCDM transplantation, which performed few injections distant from each other.

Technical Approaches for Intramuscular Transplantation

In a clinical context, a high-density injection protocol performed with precision syringes operated manually is adequate only for small volumes of muscle [20,22]. Done in that way, the method is slow and needs much concentration to ensure the depth of the intramuscular cell delivery permanently; it becomes excessively time-consuming and technically exigent for large volumes of muscle. A first attempt to alleviate this problem partially was to adapt for cell injection some laboratory dispensers initially developed for the repetitive delivery of small volumes of liquid [56]. A monosyringe dispenser became a routine instrument for SCDM transplantations in monkeys (Fig. 55.5B) [57] and was used for SCDM transplants in a patient with DMD [21]. However, its clinical use is limited to relatively small muscles and the precision needed to deliver the cells through a thick skin remains a challenge. Consequently, we determined the need to develop specific instruments for the percutaneous intramuscular injection of cells in a clinical setting. A first semimanual device was created to deliver very small quantities of cell suspension, homogeneously throughout the intramuscular trajectory of six needles at the same time, which avoids wasting in skin and hypodermis [58] (Fig. 55.7).

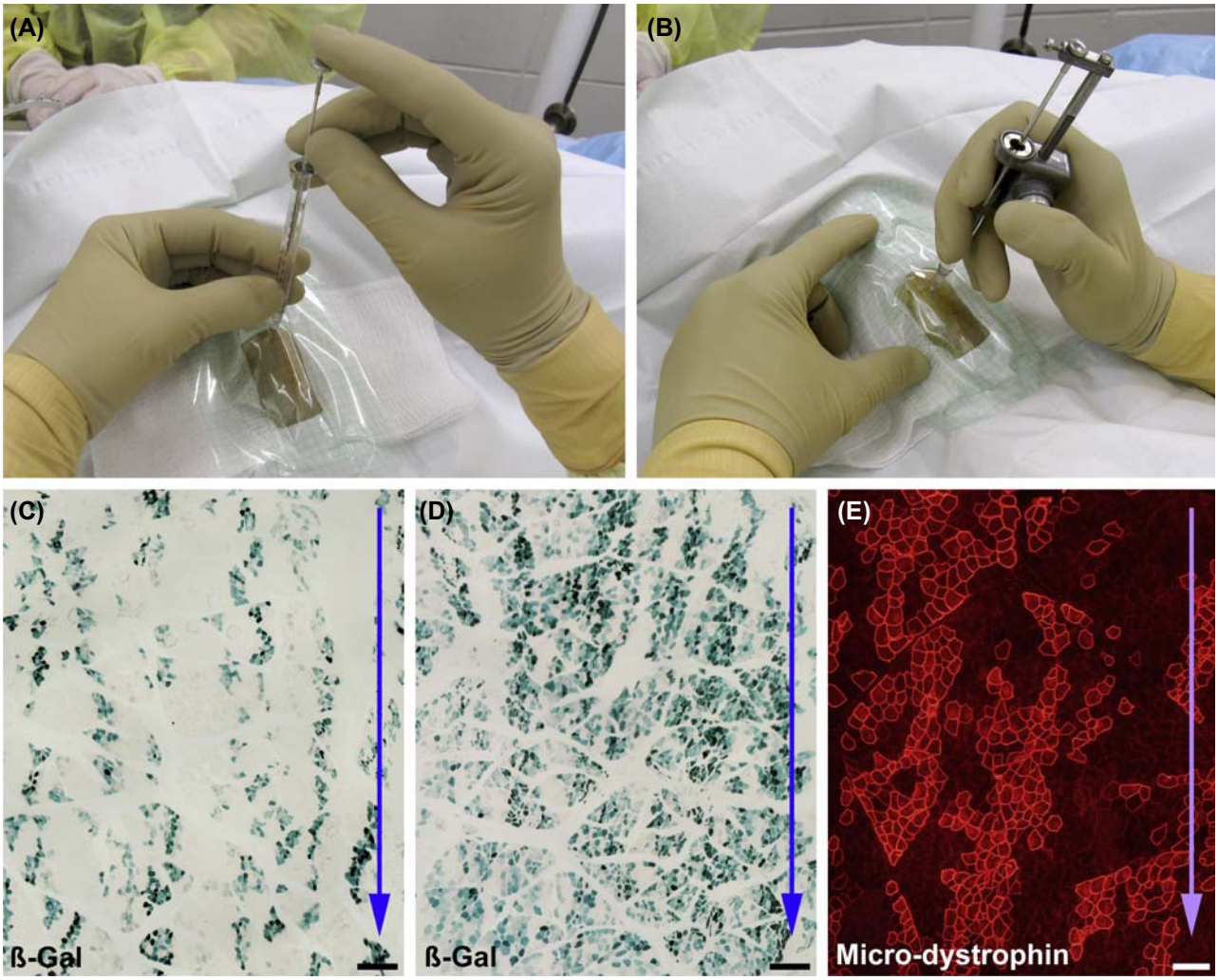

FIGURE 55.5 Examples of intramuscular satellite cell–derived myoblast (SCDM) transplantations in nonhuman primates. In most studies, SCDMs are delivered through parallel, close intramuscular injections using either a precision syringe operated manually (A) or a repeating dispenser with a precision syringe (B). As in some clinical trials (see Fig. 55.2), the density of cell injections is controlled with a sterile transparent dressing with a 5-mm grid. After 1 month, fusion of the grafted cells with the recipient's myofibers is analyzed in cross-sections of muscle biopsies using histological techniques to detect transgenic proteins that labeled the grafted cells (C–E). Histological images show cross-sections of macaque muscles grafted with transgenic SCDMs labeled either with β-galactosidase (β-Gal) (C, D) or a micro-dystrophin coupled to a peptide tag (E). Myofibers expressing donor-derived proteins are respectively detected by histochemical detection of β-Gal (C, D, *greenish blue staining*) or fluorescent immunohistological detection of the peptide tag (E). The distribution of the myofibers expressing donor-derived proteins reproduces the pattern of the original cell injection trajectories (indicated by *arrows*). The density of β-Gal–positive myofibers is higher in (D) than in (C) because the density of cell injections was higher: 25/cm^2 in (C) and 100/cm^2 in (D). Scale bars: (C, D) = 500 μm; (E) = 200 μm.

Potential Risks of the Cell Injection Procedure

A protocol of high-density injections may involve risks that need to be avoided or controlled. These risks could be local and systemic, and according to experience in nonhuman primates, they are limited to the first posttransplantation days.

Locally, a monkey's biceps brachium is swollen the first day after transplantation, but it reaches its pretransplantation diameter after 5 days [52]. This implies risks for a compartment syndrome in muscles enclosed in a rigid osteofascial space. The monkey's biceps brachium tolerates this treatment well, but for muscles such as the tibialis anterior it may be necessary to proceed cautiously in humans. The first tests in some arm muscles of a

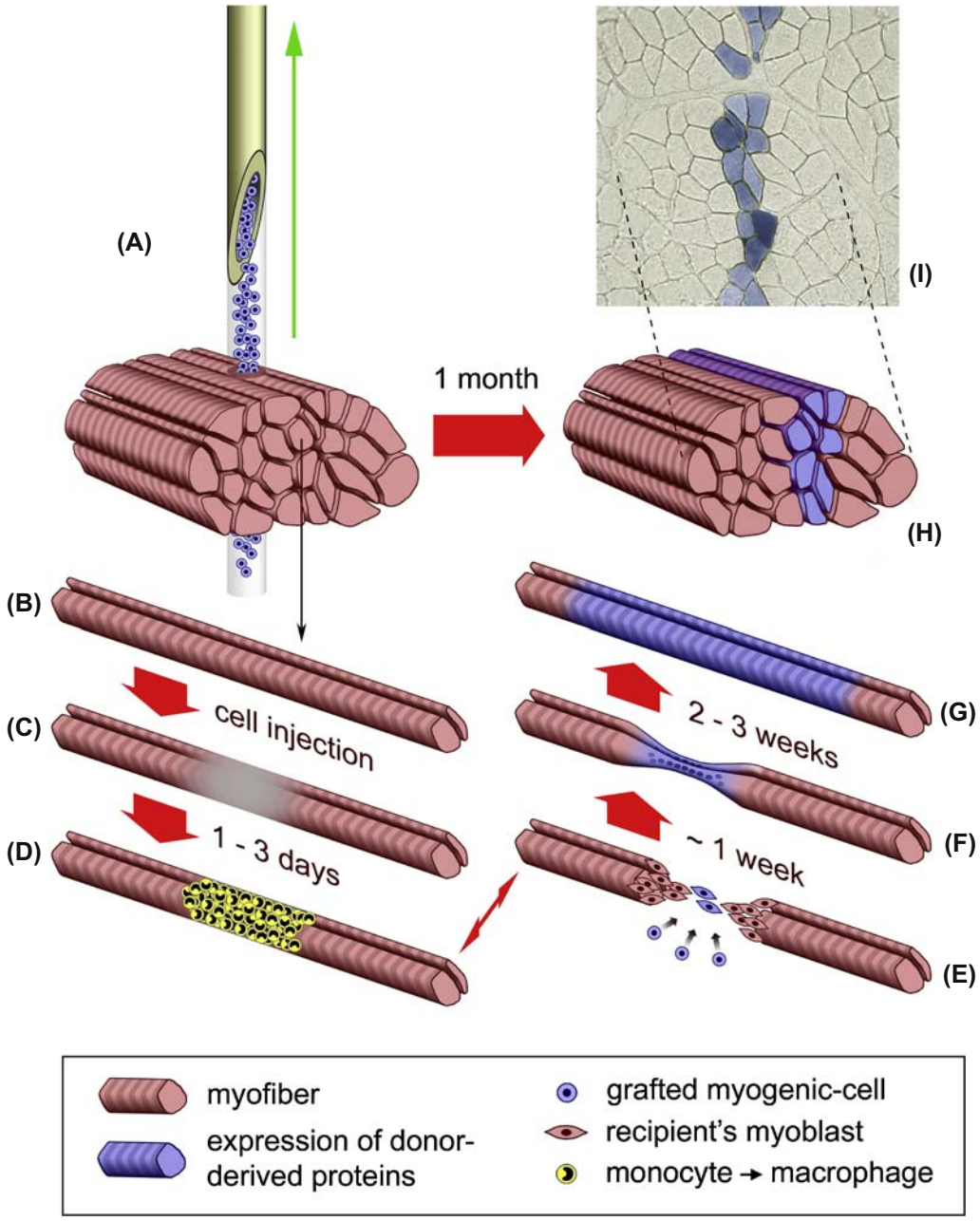

FIGURE 55.6 Representation of how the pattern of bands of engraftment is formed after the injection of satellite cell–derived myoblasts (SCDMs) in the muscles of nonhuman primates. (A) Cell injection traversing a muscle fascicle and delivering the cells homogeneously during the needle withdrawal. (B–G) The process of grafted-cell uptake in two myofibers isolated from this fascicle. Myofibers are damaged by the injection needle (B) and experience segmental necrosis (C). The necrotic region is invaded by circulating monocytes (D), which become macrophages with two main functions: phagocytosis of the necrotic debris and release of factors helping myofiber regeneration. Myofiber regeneration is done by activating the recipient satellite cells, which proliferate as SCDMs that fuse together (E). The regenerative process recruits grafted SCDMs (E). The nuclei of the grafted cells that participated in SCDM fusion are integrated in the myotubes that fill the gap led by segmental myofiber necrosis (F). Later, these graft-derived nuclei allow the expression of graft-derived proteins throughout a restricted length of the myofiber (G). This process leads to restricted regions of donor-protein expression in the fascicle (H), which are observed as bands of engraftment in cross-sections of the cell-grafted muscle (I). In this case (I), as in most SCDM transplantation studies in nonhuman primates, the grafted cells were labeled with a gene coding for β-galactosidase and the result of the graft after 1 month is evaluated through β-galactosidase expression (*greenish blue staining*) in the myofibers.

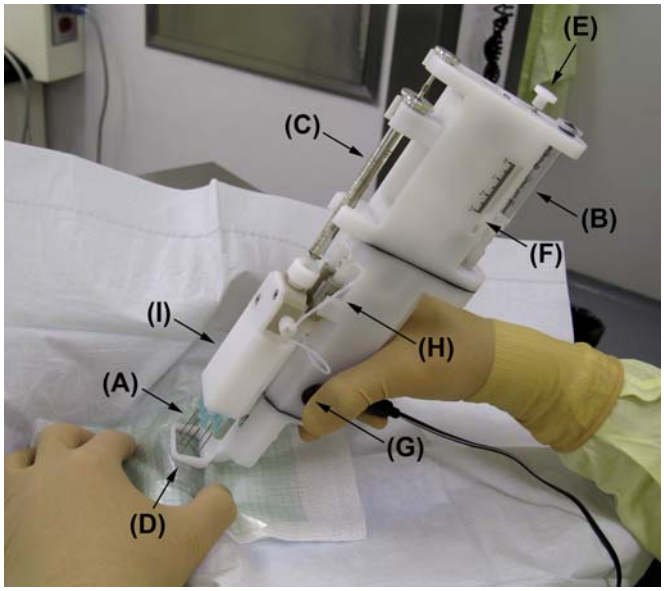

FIGURE 55.7 First device designed for the percutaneous transplantation of cells by a high-density injection protocol, shown during satellite cell–derived myoblast (SCDM) transplantation in a biceps brachium of a macaque. This prototype delivers very small quantities of cell suspension through up to six standard disposable needles (A), from a container incorporated into the device (B). From this container, the cell suspension circulates toward the injection needles during the up-and-down movements of a precision syringe (C). To deliver the cells in the muscle and not the skin, the operator can set the adjustable foot (D) to leave the needle length necessary to go through the skin and hypodermis (estimated by ultrasound analysis). The amplitude of the needles' excursion into the muscle can be set with a knob (E), using the indicator of injection depth as a reference (F). The operator then introduces the needles until the foot touches the skin surface (D); thus, the tips of the needles should be at the muscle surface. Pressing the push button (G), the needles penetrate the muscle and the precision syringe (C) is filled with cell suspension. Once the needles reach the depth of injection, they are automatically retracted to their original position and the cells are delivered into the needles' trajectory during this movement. Then the operator withdraws the needles and restarts the sequence in another position. Two valves (H) control the appropriate circulation of the cell suspension. The container, syringe, and needles are linked by a plastic support (I) that includes the flow divisor. An electromechanical system into the device's body governs the injection sequence. *Reproduced from reference Skuk D. Cell transplantation and "stem cell therapy" in the treatment of myopathies: many promises in mice, few realities in humans. ISRN Stem Cells 2013;2013:25, Article ID 582689.*

patient with DMD were well-tolerated [21], although in this case the muscles were largely replaced by fibrosis and fat tissue.

Systemically, extensive muscle damage releases intracellular metabolites such as myoglobin and potassium. This implies risks for cardiac arrhythmia in the case of severe hyperkalemia and acute renal failure in the case of myoglobinuria. However, both phenomena were not observed after high-density cell injections in the biceps brachii of monkeys [52]. Indeed, maintaining muscle damage in each transplant session under the potentially hazardous limits might prevent this problem. As an example, high-density injections throughout two biceps brachii in monkeys increased serum creatine-kinase to 2000 U/L [52] whereas the risk for acute renal failure is considered to be at 16,000 U/L [59]. This problem was not observed in a first test in a human, but the volumes of muscle injected were proportionally smaller than those used in monkeys, and as indicated earlier, these muscles were largely replaced by fibrosis and fat tissue [21].

Improving the Efficiency of Cell Injections

A lower density of cell injections is desirable, but to reach this objective, the volume of muscle expressing a therapeutic protein (e.g., dystrophin) after each single-cell injection must be increased. This could be achieved by (1) making the implanted cells to fuse with myofibers other than those around the injection trajectory, or (2)

increasing the nuclear domain of the therapeutic protein. This last possibility has rarely been investigated: only one study in *mdx* mice reported a threefold increase in the nuclear domain of dystrophin after transplantation of SCDMs overexpressing dystrophin [27].

Making the grafted cells to fuse with myofibers other than those close to the injection trajectory implies first knowing why this happens. Two factors were previously mentioned to explain why the grafted SCDMs fuse mostly with the myofibers in the trajectory of injections: (1) the grafted SCDMs lack the capacity to move through the tissue and therefore remain in the injection trajectories to fuse with the neighboring myofibers; and/or (2) the grafted SCDMs have the capacity to move through the tissue but myofiber regeneration is mandatory to attract the transplanted SCDMs to fuse with them, and in a normal muscle this occurs only in the myofibers damaged by the injection.

Addressing the first factor, various studies wanted promotion of the intramuscular diffusion of the grafted SCDMs by inducing in them the secretion of enzymes that degrade the extracellular matrix [60–63]. Several of these studies improved the migration capacity of SCDMs under experimental conditions in vitro and in vivo in mice, and even enhanced the transplantation outcome. However, the first tests in monkeys were disappointing and suggested that an increasing migration capacity in the implanted SCDMs does not augment the success of their transplantation per se [63]. Subsequent experiments in nonhuman primates demonstrated that grafted SCDMs have an intrinsic capacity to migrate several millimeters into the muscle, but that they migrate essentially to fuse with regenerating myofibers in a mechanically damaged tissue [64].

Addressing the second factor, other experiments increased the number of regenerating myofibers to favor the uptake of the grafted SCDMs. Local injection of myotoxins such as phospholipases derived from snake venoms [17,65] and local anesthetics [66,67] are efficient in small rodents for this purpose. Intense muscular exercise produced extensive myofiber necrosis and almost doubled the success of SCDM transplantation in dystrophic mice [68]. Conjointly, inhibiting the proliferation of the recipient's satellite cells favors the participation of the grafted SCDMs in myofiber regeneration. This is achieved in mice by exposing the recipient muscles to high doses of ionizing radiation before cell transplantation [17,69]. Cryoinjury of recipient muscles necroses myofibers and satellite cells and has been used in mice as a pretreatment to favor the implanted cells [30,31,41].

In nonhuman primates, the coinjection of SCDMs and myotoxic phospholipases improved the outcome of SCDM transplantation but only when SCDMs and myotoxins were highly concentrated in the muscle [52,70]. A method that was especially efficient in inducing extensive myofiber regeneration in the large muscles of nonhuman primates, with the consequent increase of the SCDM engraftment in extensive zones, was intramuscular electroporation [71]. In the event that one of these methods could be translated to the clinic, it should be taken into account that increasing muscle damage would reduce the volume of muscle to be treated in one session, considering the risk for a rhabdomyolysis-like phenomenon as mentioned earlier.

CELL-GRAFT SURVIVAL

Once a good delivery of the appropriate cells is obtained, their survival in the recipient must be ensured. The posttransplantation survival of myogenic-cells should be analyzed for two periods: the initial (the first week) and the long-term (the life span of the patient).

Initial Survival

There is a consensus that an important mortality occurs among grafted SCDMs rapidly after their intramuscular implantation, essentially within the first week posttransplantation. This was deduced by the progressive loss of different grafted-cell markers after SCDM transplantation in mice [72–74]. In addition, morphological evidence of apoptosis and necrosis was found among the grafted SCDMs early after implantation [74].

This early cell death does not prevent the outcome of SCDM transplantation, because not all cells die, and in some mouse experiments the proliferation of the surviving cells compensated totally [74] or partially [73] the cell death. This initial cell death is not well-understood, and studies approaching this topic show contradictions, probably because of methodological differences [74]. An early study in mice blamed cells of the acute inflammatory reaction for killing the implanted SCDMs [72], but later experiments found no evidence of neutrophil, macrophage, or natural killer cell responsibility in this death [75]. It was also postulated that the survival of the graft could be caused by a special small subpopulation of cells that specifically avoid early cell death and proliferate greatly [73]. Other mechanisms proposed to be responsible for causing apoptosis among SCDMs grafted in mice were hypoxia [76] and anoikis [77].

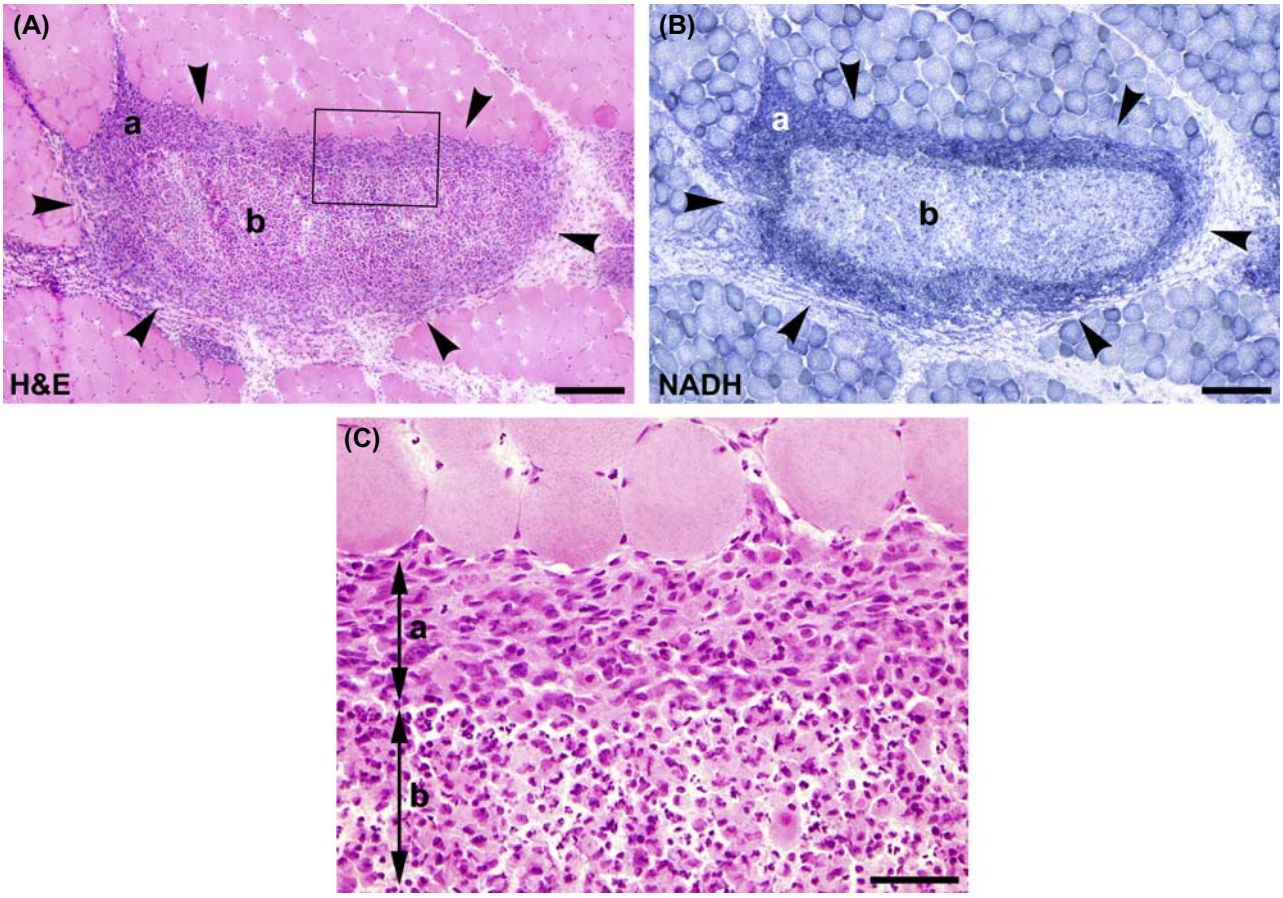

FIGURE 55.8 Ischemic necrosis in an intramuscular accumulation of grafted satellite cell–derived myoblasts 1 day after their transplantation in the skeletal muscle of a nonhuman primate. *Arrowheads* in (A) circumscribe the intramuscular accumulation of grafted cells in a section stained with hematoxylin-eosin (H&E). Two regions with different staining characteristics can be observed in this cell accumulation: a darker peripheral ring (a) and a paler core (b). In a serial section (B) stained for histochemical detection of the oxidative enzyme nicotinamide adenine dinucleotide reduced diaphorase (NADH), the two regions are more clearly delimitated: a peripheral ring strongly stained (a: living cells with oxidative activity) and a central region almost devoid of oxidative reaction (b: necrosed cells with a loss of oxidative activity). A detail of this accumulation of grafted cells (*rectangle* in [A]) is shown at higher magnification in (C). (C) The peripheral layer (a) is composed of cells with normal characteristics, whereas the core (b) has smaller cells with pyknosis, karyorrhexis, and in several cases, loss of cytoplasmic staining. Scale bars: (A, B) = 200 μm; (C) = 50 μm. *Reproduced from reference Skuk D. Cell transplantation and "stem cell therapy" in the treatment of myopathies: many promises in mice, few realities in humans. ISRN Stem Cells 2013;2013:25, Article ID 582689.*

Otherwise, a well-determined mechanism of rapid grafted-cell death detected in monkeys is the ischemic necrosis of the central part of the intramuscular accumulations of grafted SCDMs (Fig. 55.8) [78]. Injected cells form avascular accumulations and their survival depends on the oxygen and nutrients diffusing from the surrounding tissue. Because this diffusion is limited, only a peripheral cell layer of about 100–200 μm survives. For this reason, the amount of central necrosis depends on the size of the grafted-cell clusters [78].

Long-term Survival

The principal threat to the long-term survival of SCDM grafts is acute rejection in inadequately immunosuppressed allogeneic transplantation, as confirmed in nonhuman primates [79]. Acute rejection in the context of SCDM transplants was extensively studied in mice since the first description of lymphocyte infiltration and grafted SCDM disappearance soon after allogeneic transplantation [80]. Subsequent studies identified CD8+ and CD4+ lymphocytes in these infiltrates [81,82], and expression of interleukin-2 receptors, T-helper 1 cytokine, and granzyme B confirmed that these were activated lymphocytes [83,84]. Incompatibility in minor antigens can also trigger acute rejection of hybrid myofibers, as observed in mice after syngeneic transplantation into females of SCDMs obtained from males [85].

However, observations in mice were not useful in the analysis of immune responses and acute rejection in clinical trials of SCDM transplantation [20,21]. For this purpose, studies in nonhuman primates defined the histological features of acute rejection of hybrid myofibers [79]. Independent of the way to induce acute rejection of the hybrid myofibers (withdrawal of immunosuppression, low immunosuppression, or progressive reduction of immunosuppression) and of the posttransplantation period in which rejection occurs, graft loss was associated in all cases with myositis characterized by dense focal accumulations of CD8+ and CD4+ lymphocytes and a more diffuse component of macrophages [79]. Typically, these lymphocyte accumulations were mainly endomysial, totally or partially surrounding some myofibers and often invading them. Some mechanisms by which the immune system eliminates the hybrid myofibers were identified: There was evidence of sarcolemmal damage in the myofiber regions invaded by lymphocytes, together with myofiber necrosis and caspase 3 activation in some of the myofibers attacked by CD8+ lymphocytes [86].

Ensuring Cell Survival in the Recipient

Early cell death among grafted SCDMs is misunderstood. For that reason, it has not been prevented efficiently. However, because this death does not devastate the population of implanted SCDMs, which seems well-restored by the proliferation of the surviving cells, the only potential benefit of inhibiting it would theoretically be a reduction in the number of cells to be injected [87]. This would not be totally insignificant, because it has been estimated in nonhuman primates that the number of SCDMs to be injected per cubic centimeter of muscle, using matrices of 100 injections/cm^2, is between 10×10^6 and 100×10^6 cells [55]. Conversely, massive ischemic central necrosis can be prevented or reduced by ensuring the formation of accumulations of grafted SCDMs in which most cells are within 100–200 μm of the surrounding tissue [78].

Acute rejection, on the other hand, precludes the survival of an SCDM allograft. Whereas acute rejection is efficiently controlled by pharmacological immunosuppression, careful selection of the drugs is required for SCDM transplantation because some of them kill and/or inhibit differentiation of the grafted SCDMs [6]. Because the best results of SCDM allotransplantation in mice were reported using tacrolimus for immunosuppression [17], this drug became the immunosuppressant of choice for SCDM allotransplantation in monkeys [57] and consequently in humans [20–23]. The first clinical trial of SCDM transplantation with tacrolimus immunosuppression involved follow-up of only 1 month [20,22], but a patient in whom tacrolimus was continued for 18 months showed preservation of donor-derived dystrophin during that time [21].

Because pharmacological immunosuppression has severe secondary effects, a main objective in clinical transplantation is to create immune tolerance: that is, long-term specific unresponsiveness to grafts with preservation of immune reactions against other foreign antigens. In mice, a transient immunosuppression can be sufficient to develop immune tolerance to SCDM allotransplants in some strains or to delay acute rejection for months in others [30,88] but in nonhuman primates, immunosuppression withdrawal causes rapid acute rejection of the hybrid myofibers [79,86]. This is another example of how immune tolerance is more easily obtained in mice than in monkeys or humans [89]. Some protocols to develop immune tolerance were tested for SCDM transplantations in mice. Central tolerance via mixed chimerism [90,91] was more successful than peripheral tolerance combining donor-specific transfusion and administration of anti-CD154 antibodies [92]. However, central tolerance in the context of myogenic-cell allotransplantation may not include neoantigens appearing in the hybrid myofibers and would also need peripheral-tolerance mechanisms to avoid acute rejection [93].

Another approach to avoiding immunosuppression in this context is the autotransplantation of SCDMs genetically corrected ex vivo [94]. Tests in mice with different viral vectors or transfection approaches to introducing the therapeutic transgene in SCDMs were considered to be promising for future development with this approach [94], and the feasibility of this strategy was also tested in macaques [54]. However, some of these tactics leave open the possibility of a rejection owing to incompatibility of minor antigens, essentially neoantigens from the product of the transgene incorporated in the cells [95].

CONCLUSIONS

To reach a therapeutic objective, cell transplantation basically needs three conditions: a cell that meets the properties required for the treatment, a method of cell administration that ensures that the treatment could be clinically relevant, and conditions to ensure the survival of the graft throughout the life of the recipient. So far, SCDMs are the only myogenic cells that have passed a clinical test of cell transplantation in skeletal muscles, restoring dystrophin in

variable amounts of myofibers in patients with DMD. Intramuscular implantation through a protocol of high-density injections is the only method that has produced relevant percentages of hybrid myofibers in humans. Finally, a tacrolimus-based immunosuppression is the only method that has been successfully tested in nonhuman primates and humans to ensure the survival of the hybrid myofibers produced by SCDM allografts.

Some important challenges remain to render this technique applicable in clinics with any expected benefit for patients. A main challenge is to enhance the success of SCDM transplantation further while reducing the density of cell injections. Another challenge is a main problem in the global context of transplantation: that is, minimizing as much as possible the toxicity of the methods needed to control graft rejection. This could be achieved by refining immunosuppressive protocols or developing efficient approaches to immune tolerance. Cell transplantation also offers the alternative of manipulating cells before transplantation to avoid immunosuppression, which opens the possibility of autotransplantation of genetically corrected cells. Finally, the capacity to restore the functional parenchyma in skeletal muscles that have degenerated to fibrosis and/or fat substitution remains unsolved and barely studied. However, this would be the only possibility of restoring muscle function in degenerative muscular pathologies such as DMD.

List of Abbreviations

DMD Duchenne muscular dystrophy
SCDM Satellite cell−derived myoblast

Glossary

Myoblast Proliferating mononuclear progenitor cell that fuses with analogous cells to form myotubes: that is, syncytial multinucleated cells with central nuclei, a small diameter, and few myofibrils, which under adequate conditions (essentially motor innervation) precedes myofiber formation.

Myofiber Syncytial multinucleated cells of cylindrical shape that are the terminally differentiated contractile units of the skeletal muscle, composed essentially of contractile proteins (myosin and actin) organized in sarcomeres and that have a specialized system for excitation−contraction coupling.

Satellite cell Mononuclear myogenic precursor cell located in the periphery of myofibers, between the basement membrane and the sarcolemma, where it remains quiescent while the muscle myofiber remains intact, and is activated when the myofiber is damaged, reentering the cell cycle to: (a) generate myoblasts to regenerate the myofiber and (b) give rise to other satellite cells to maintain the pool of muscle progenitors.

Satellite cell−derived myoblasts Myoblasts involved in postnatal myofiber regeneration that are generated by the activation and proliferation of satellite cells, either in vivo, in response to a myofiber injury that causes segmental or total necrosis and therefore needs to be repaired or in vitro, during the cell culture of skeletal muscle biopsies.

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CHAPTER

56

Islet Cell Transplantation

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INTRODUCTION

Background

Diabetes is a disease that results from impaired glucose metabolism. Approximately 90% of diabetes is caused by a defect in insulin production and/or use (type 2 diabetes mellitus [T2DM]), whereas the more severe form, type 1 diabetes mellitus (T1DM), is caused by a complete loss of insulin-producing β cells within the islets of Langerhans of the pancreas. Diabetes affects more than 200 million patients worldwide and is projected to afflict at least 5% of the global adult population by 2025 [1]. As the incidence of diabetes has increased, the cost of treating these patients has skyrocketed, consuming between 7% and 13% of health care expenditure in developed countries [2]. Since the discovery of insulin in 1921, diabetes has become a treatable condition and the life expectancy of patients with diabetes has been greatly improved. However, even with diligent blood glucose monitoring and insulin administration, metabolic abnormalities associated with diabetes often lead to many chronic secondary complications, including nephropathy, retinopathy, peripheral neuropathy, coronary ischemia, stroke, amputation, erectile dysfunction, and gastroparesis [3]. In the United States, patients with diabetes represent 8% of those who are legally blind and 30% of all patients receiving dialysis caused by end-stage renal disease, and 20% of all patients receiving kidney transplants [3]. The Diabetes Control and Complications Trial (DCCT) was conducted to determine whether intensive blood glucose regulation by frequent insulin injection or pump could prevent these long-term complications in patients with diabetes [4–6]. Results from the DCCT and subsequent Epidemiology of Diabetes Interventions and Complications study clearly demonstrated that this approach improved but did not normalize glycosylated hemoglobin (HbA1C) levels and significantly protected against cardiovascular disease, nephropathy, neuropathy, and retinopathy [5–8]. However, the consequence of improved glycemic control was a threefold increased risk for serious hypoglycemic reactions leading to recurrent seizures and coma [5,9]. Improvements in the size and sensitivity of insulin pumps have increased their utility and provide possible alternatives for stabilizing glycemic control in diabetic patients [10]. Insulin pump therapy can improve HbA1C levels compared with multiple daily injections of insulin, but pumps may malfunction and thus still necessitate frequent blood glucose monitoring by the user, and they have not resulted in glycemic stabilization rates achieved in islet transplantation [11,12].

Although advances in the formulation, half-life, and administration of insulin have markedly improved the quality of life and long-term survival of patients with diabetes, it has long been recognized that the restoration of an adequate islet mass would provide maximum benefit to diabetic patients, leading to a true physiological correction of the diabetic state. In the early 1960s, great advances were made in the field of renal transplantation owing to improved immunosuppressive therapies (azathioprine and corticosteroids), which prompted the first attempts in whole-pancreas transplantation [13,14]. First introduced by Kelly and Lillehei in 1966, early attempts were associated with high mortality rates and poor graft survival, with less than 3% graft function at 1 year after transplant [15]. The risk profile and long-term outcomes in whole-pancreas transplantation have been greatly improved by improvements in surgical technique, including portal venous and enteric endocrine drainage and steroid-free maintenance immunosuppression [16,17]. To date, more than 25,000 pancreas transplants have been performed worldwide for end-stage renal disease (simultaneous kidney pancreas or pancreas after kidney transplantation) or less frequently for severe hypoglycemic unawareness (pancreas transplant alone). Data collected in the International

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Pancreas Transplant Registry showed that only 50% of patients who have undergone pancreas-alone transplantation remain insulin independent at 5 years despite improvements in surgical technique and immunosuppression [18–20]. Also, less than 30% of the approximately 6000 cadaveric pancreata donated each year are transplanted because of strict donor criteria and requirements for short cold ischemic time [18,21]. The surgical risks and requirement for lifelong immunosuppression have reserved pancreas-alone transplantation only for diabetic patients with the most severe and life-threatening disease, despite strong evidence that the procedure can prolong life, reverse established nephropathy, and improve quality of life. Because the major surgical complications in whole-pancreas transplantation are related to the exocrine function of the pancreas, which is not necessary to restore euglycemia in diabetic patients, it has long been recognized that β-cell replacement could be achieved with implantation of isolated pancreatic islets. This approach involves transplantation of a cellular graft that would be implanted using minimally invasive techniques; thus, it would avoid the risks associated with major surgery, resulting in a more widely available treatment for patients with diabetes.

History of Islet Transplantation

The concept of islet transplantation actually preceded the discovery of insulin in 1921 by nearly 30 years (Fig. 56.1). In 1893, physicians in Bristol, England, attempted to treat a young boy who had diabetic ketoacidosis by transplanting fragments of a freshly slaughtered sheep's pancreas [22]. Although the graft ultimately failed in the absence of immunosuppression, there was temporarily improvement in the boy's state, which suggested that cells within the pancreas could stabilize diabetes. After the discovery of insulin, it was thought that exogenous insulin replacement would be an effective treatment for patients with T1DM, and therefore islet transplantation was not actively pursued. However, as insulin therapy transformed T1DM from an acute health crisis to a chronic disease, it became apparent that insulin injections could not prevent the onset of debilitating and life-threatening secondary complications. Because the first series of whole-pancreas transplants in the late 1960s were associated with poor morbidity and mortality, isolated islet transplantation gained renewed interest [23]. The first successful islet isolations and subsequent transplantation into chemically induced diabetic rodents were pioneered by

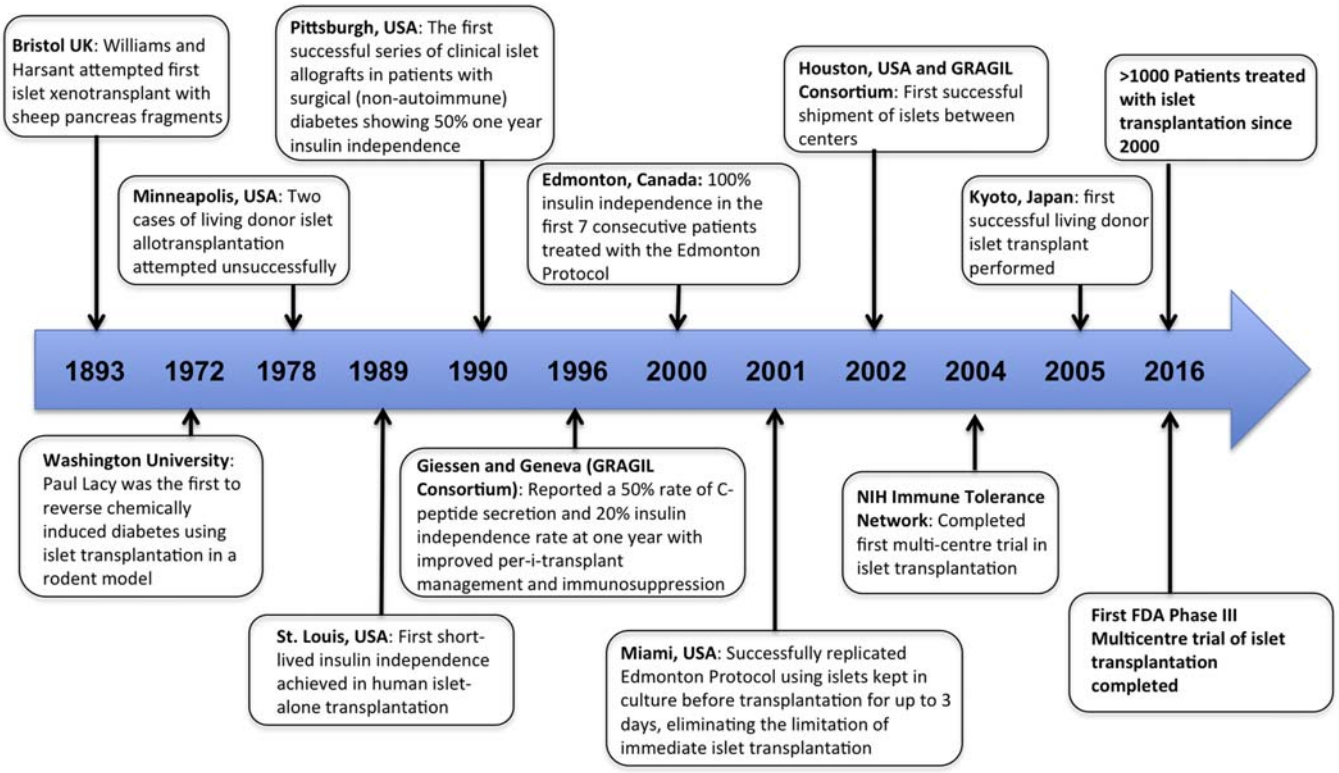

FIGURE 56.1 Timeline of notable advances in the history of islet transplantation. *GRAGIL*, Groupe Rhin–Rhône–Alpes et Genève Pour la Greffe d'Îlots de Langerh.

Dr. Paul Lacy at Washington University in St. Louis, which immediately sparked interest in implementing clinical trials [24–27]. Although euglycemia was routinely obtained in animal models of islet transplantation, clinical islet transplantation struggled to find success for most of the 1970s and 1980s. During that time, unpurified islets were infused into the portal vein, which led to many serious complications including portal vein thrombosis, portal hypertension, and disseminated intravascular coagulation [28]. While working in Lacy's group, Dr. Camillo Ricordi developed the "automated method" for high-yield islet isolation in 1989 [29]. This represented a major turning point in the field and led to the report that Lacy's group had achieved short-lived insulin independence in a patient with T1DM who had received an islet graft after a previous kidney transplant [30]. The following year, the group led by Ricordi at the University of Pittsburgh reported the first series of clinical islet allografts that demonstrated improved insulin independence rates of 50% at 1 year in subjects who underwent cluster islet-liver transplants for abdominal malignancies in the setting of surgically induced (nonautoimmune) diabetes [29,31]. Although this represented a major advance in the field of islet transplantation, these results could not be reproduced in patients with T1DM, the important patient population in need of β-cell replacement [32]. In the late 1990s, the European Groupe Rhin–Rhône–Alpes et Genève Pour la Greffe d'Îlots de Langerhans (GRAGIL) consortium reported the first modestly successful insulin independence rates of 20% at 1 year in patients with T1DM, which could be attributed to improved peritransplant management and immunosuppressive drug regimens [33]. Because the results from Pittsburgh and the GRAGIL consortium were obtained in patients who had previously received a kidney transplant, there was no additional risk in terms of immunosuppression to patients after receiving an islet graft [32,33]. An international registry held in Giessen, Germany, has maintained a comprehensive record of previous clinical attempts at islet transplantation globally, and of the total world experience of over 450 attempts at clinical islet transplantation before 2000, less than 8% of subjects achieved insulin independence [34]. After 3 decades of research, the 1-year insulin independence rates in clinical islet transplantation were still too low to justify the risks associated with portal infusion and lifelong immunosuppression in most patients with T1DM [33–37].

The Edmonton Protocol

Shapiro and colleagues at the University of Alberta developed a protocol in 1999 that was designed for patients with "brittle diabetes" who experienced extreme difficulty in managing blood glucose levels ("glucose lability") and/or severe hypoglycemic unawareness [38]. The so-called "Edmonton Protocol" was unique compared with previous attempts in clinical islet transplantation in its high targeted islet mass, with a mean of approximately 13,000 islet equivalents (IE)/kg recipient body weight, often derived from two (or occasionally more) fresh islet preparations and in its immunosuppression strategy, with emphasized avoidance of corticosteroids and the use of potent immunosuppression with combined sirolimus, tacrolimus and anti-CD25 antibody to protect against rejection and recurrent autoimmunity [38]. This approach led to dramatic improvements in islet allograft survival; all of the first seven patients achieved sustained independence from insulin [38]. Almost 300 consecutive patients have received islet transplants at the University of Alberta since 1999, and the 1-year insulin independence rate remains steady at approximately 80% after completed transplants (<13,000 IE/kg). Results obtained at the University of Alberta have been replicated at other centers including an international multicenter trial through the Immune Tolerance Network, but each center's success has varied greatly depending on its previous experience and skill in islet isolation and immunosuppressive management [39,40]. The Miami group demonstrated that islets can be cultured for up to 3 days before transplant or shipped and transplanted at a remote facility (Houston) with similar success as freshly isolated islets when transplanted using Edmonton-like immunosuppression [41,42] The GRAGIL Network (a Swiss–French consortium) also demonstrated the benefits of centralized islet processing facilities that can service a broader network of centers throughout Europe [33,43].

Based on the success of the Edmonton group, islet transplantation has been funded in Alberta, Canada, as accepted clinical standard of care since 2001. Progress in this area has been challenging in the United States, but large registration trials have been completed, which should facilitate Biological Licensure by the Food and Drug Administration, and therefore reimbursement, which will make a significant difference to the availability of islets for transplantation in that country [44,45]. The success of clinical islet transplantation has encouraged many centers around the world to implement a program, and since 2000 more than 1000 patients have been transplanted using variants of the Edmonton Protocol in almost 50 centers worldwide [46].

Despite this success, the requirement for lifelong immunosuppression in islet-alone transplantation has restricted its availability to patients with T1DM and severe hypoglycemia or glycemic lability. The benefit of islet transplantation in patients with T2DM has not been determined, because many of these patients are overweight and/or insulin resistant and thus would require a large islet mass to meet metabolic demands. Most patients require two

or occasionally three islet implant procedures to achieve insulin independence, although insulin independence after single donor infusion was reported in a cohort of patients at the University of Minnesota [47,48]. In Edmonton, data indicate that C-peptide secretion (>0.5 ng/mL) is maintained in almost 70% of patients in the long term (up to 18 years), and through this, patients derive ongoing benefit in terms of stabilization of glycemic control without hypoglycemic reactions. Overall, a quarter of patients remain completely insulin free at 5 years, but this rises to 50% in patients treated in newer T-depleting and antiinflammatory induction protocols. Although the cause of the discrepancy between insulin independence and maintenance of C-peptide status is not fully understood, it is likely that multiple events hinder graft function and survival over time. Whereas rejection (acute or chronic) and recurrent autoimmunity may be responsible for graft loss, it is probable that other, nonimmune-mediated damage occurs, such as chronic toxicity from tacrolimus. Perhaps the most important component of decaying graft function over time is the concept of islet "burnout" from constant metabolic stimulation, because only a marginal mass of islets actually engraft in most subjects. In clinical islet transplantation, the risks for malignancy, posttransplant lymphoma, and life-threatening sepsis have been minimal, but fears regarding these complications limit broader application in patients with less severe forms of diabetes, including children [46]. Moreover, a number of immunosuppression-related side effects have been encountered, including dyslipidemia, mouth ulceration, peripheral edema, fatigue, ovarian cysts, and menstrual irregularities in female subjects, which can be dose or drug limiting in some patients [49]. Thus, although dramatic improvements in outcomes after islet transplantation have been observed, extensive refinements in clinical protocols are needed to improve safety and enhance success with single donor islet infusions.

CLINICAL ISLET TRANSPLANTATION

Patient Assessment and Selection

Clinical islet transplantation is associated with a series of potential risks, including procedural complications such as bleeding or portal vein thrombosis, or those associated with lifelong immunosuppression, i.e., infection or malignancy. For these reasons, patients selected as islet recipients must have severe, life-threatening diabetic complications that justify the risks of transplantation. Two T1DM patient populations have been identified as suitable candidates for islet transplantation: individuals who experience frequent, severe, and recurrent hypoglycemic unawareness, and those with highly unstable blood glucose control despite an optimized insulin regimen (glycemic lability).

When patients are evaluated for islet transplantation, their metabolic status and diabetes-related secondary complications should be carefully characterized so that those who would receive the greatest benefit despite the requirement for lifelong immunosuppression are selected. First and foremost, islet transplantation is reserved for patients with C peptide−negative (<0.3 ng/mL) T1DM. Recipients with an elevated body mass index (>30 kg/m^2) or those greater than 90 kg are generally excluded, because their metabolic demand may not be met by the transplanted islet mass. As mentioned previously, indications for islet-alone transplantation include severe hypoglycemic unawareness and/or glycemic lability. To assess these symptoms, Ryan et al. developed an objective scoring system to measure the severity of both hypoglycemia (the HYPO score), and the (LI), which is based on changes in blood glucose over time [50]. Selection criteria for islet-alone transplantation include a HYPO score greater than 1047 (90th percentile), LI > 433 mmol/L^2/h · $week^{-1}$ (90th percentile), or a composite with the HYPO score greater than 423 (75th percentile) and LI greater than 329 (75th percentile) [51]. Because patients with poor diabetes compliance or an inadequate baseline insulin regimen are likely to benefit from an improved design of insulin dosing regimens, patients selected for transplant should have a plasma HbA1C less than 10%. In an effort to reduce the risk of serious procedural and immunosuppressive drug-related complications, the patient's cardiac and renal function should be carefully assessed. Selected recipients should have adequate cardiac function including blood pressure less than 160/100 mmHg, no evidence of myocardial infarction in the 6 months before assessment, no angiographic evidence of noncorrectable coronary artery disease and left ventricular ejection fraction greater than 30% as measured by echocardiogram. To eliminate patients who are better candidates for simultaneous kidney−pancreas transplantation or those who may experience adverse renal function as a result of tacrolimus, selected recipients should have no evidence of macroscopic proteinuria (<300 mg/24 h) and a calculated glomerular filtration rate greater than 80 (>70 in females) mL/min/1.73 m^2. Proliferative retinopathy should be stabilized before transplantation, because acute correction of glycemic control may lead to accelerated retinopathy. Finally, to reduce

the risk of antibody-mediated graft rejection, potential recipients should be screened for panel reactive antibody (PRA) assays. It remains unknown whether patients with elevated initial PRA will have worse long-term islet function if they have a negative cross-match to the specific donor cells.

Islet Transplantation Procedure

Although several locations have been tested as potential implantation sites for islet grafts, the high level of graft function and ease of delivery associated with infusion into the portal circulation of the liver have led to this being the transplantation site of choice in clinical protocols [27]. There are two accepted approaches for implanting purified islets into the liver by way of the portal vein. Whereas surgical laparotomy and cannulation of the portal vein was most often used in the early islet transplant programs, current protocols routinely employ the percutaneous transhepatic approach to implant donor islets in cadaveric islet transplantation (Fig. 56.2A) [51]. Compared with surgical laparotomy, this procedure is minimally invasive and thus can be performed using local anesthesia combined with opiate analgesia and hypnotics given as premedication. Access to the portal vein is achieved by percutaneous transhepatic approach using a combination of ultrasound and fluoroscopy to guide the radiologist. A branch of the right portal vein is cannulated and a catheter is positioned proximal to the confluence of the portal vein, which is confirmed with a portal venogram [52]. The risk for portal vein thrombosis is reduced by inclusion of unfractionated heparin (70 U/kg) in the islet preparation. Islets are then infused aseptically into the main portal vein under gravity, with regular monitoring of portal venous pressure (by an indirect pressure transducer) before, during, and after the infusion. An ultrasound examination should be performed at 1 day and 1 week after transplant to rule out intraperitoneal hemorrhage and confirm that the portal vein is patent and has normal flow.

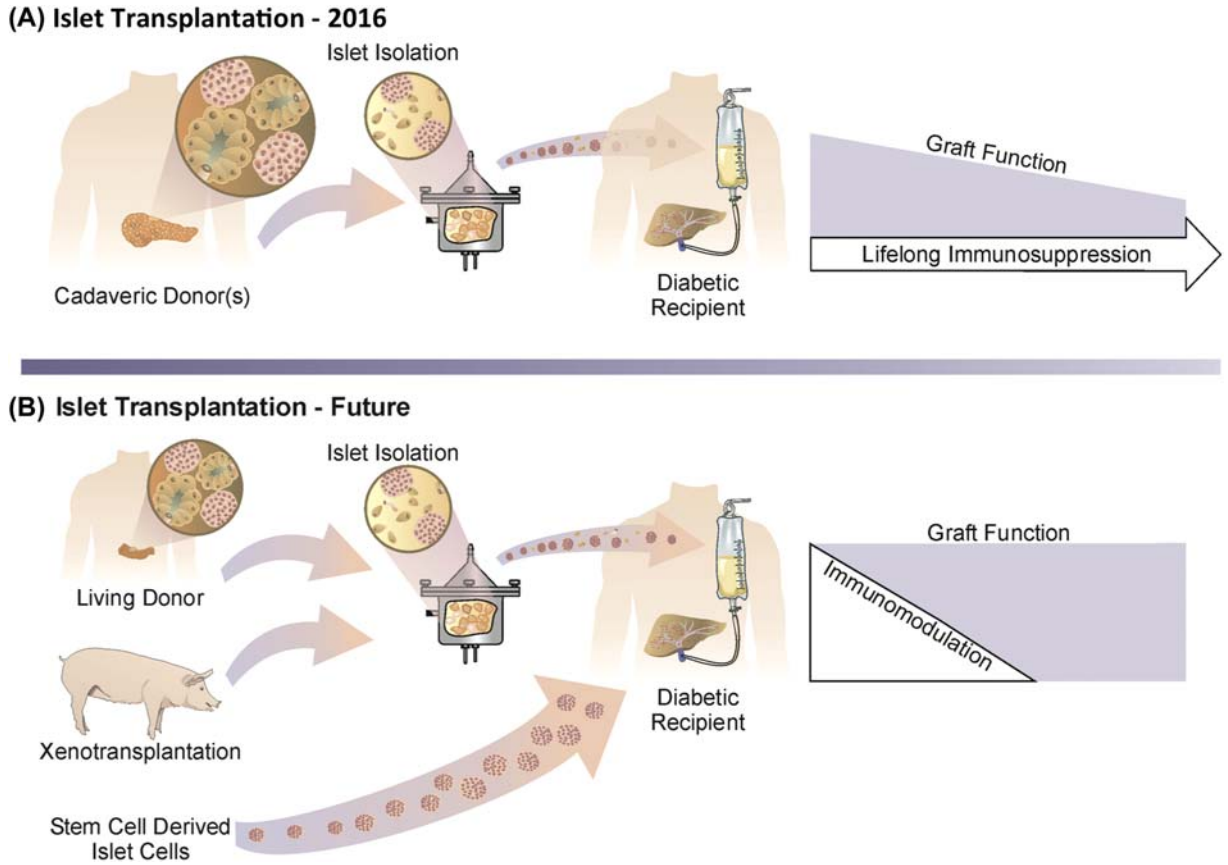

FIGURE 56.2 The islet transplant procedure: present and future. Islet transplantation, in its current form (A), has provided insulin independence in most diabetic patients at 1 year after transplant, but this procedure is limited by the availability of suitable cadaveric donors and the requirement for lifelong immunosuppression. In the future (B), islet transplantation could be made available to a broader range of diabetic patients through the use of alternative tissue sources such as living donors, xenogeneic donors, or stem cell–derived β cells. Also, as novel immunomodulatory therapies are identified, tolerance induction strategies can be developed that will prolong graft function and allow for the reduction or complete withdrawal of immunosuppressive drug therapy.

If the skills of a local expert interventional radiologist are unavailable or if a large hemangioma is present on the right side of the liver at risk for puncture and bleeding if the percutaneous approach were to be used, surgical laparotomy and cannulation of a mesenteric venous tributary of the portal system should be considered. In this situation, complete surgical control is in place to prevent uncontrolled bleeding. Another advantage includes the potential for use of a dual-lumen catheter for cannulation of a mesenteric vein (i.e., dual-lumen 9-French Broviac line), which allows for continuous monitoring of portal pressure during islet infusion. Still, this surgical approach should be considered only when the percutaneous transhepatic approach cannot be used, because it presents several major disadvantages, including the requirement for a surgical incision, formation of adhesions, and the risk for wound infection and wound herniation, which may be exacerbated when the drug sirolimus is used after transplant, because this drug interferes with wound healing.

Risks to the Recipient

Surgical Complications

There are two potentially serious procedural complications in islet transplantation: bleeding from the catheter tract created by the percutaneous transhepatic approach, and portal vein thrombosis, particularly when large volumes of tissue are infused. Adverse bleeding events were noted early in the development of the Edmonton program, but these have been almost completely avoided with the routine use of effective methods to seal and ablate the transhepatic portal catheter tract on egress when the catheter is withdrawn [53]. The combination of coils and tissue fibrin glue (Tisseel) was used previously, but it has been replaced by Avitene paste (1 g Avitene powder mixed with 3 mL radiological contrast media and 3 mL saline; approximately 0.5−1.0 mL of this paste is injected into the liver tract) [54]. The use of purified islet allograft preparations has not resulted in main portal vein thrombosis in the Edmonton program, but thrombosis of a right or left branch or peripheral segmental vein has been encountered in approximately 5% of patients. Other rarely observed procedural side effects have included fine-needle gallbladder puncture, which has been avoided with the use of ultrasound-guided transhepatic portal venous access. Rarely, arteriovenous fistulae (which may require selective embolization) or hepatic steatosis have been observed [55].

Immunosuppressive Therapy and Complications

Islet transplantation for T1DM represents a unique challenge in immunosuppression, because both alloimmunity and islet-specific autoimmunity must be effectively controlled to preserve graft function. An additional important consideration is that many of the immunosuppressive agents used in solid organ transplantation since the 1960s, particularly corticosteroids, are known to be toxic to islets. Previously in the Edmonton Protocol, the induction agent daclizumab (anti-CD25 [interleukin (IL)-2R] antibody) was used, but this was replaced by basiliximab when daclizumab was no longer available for clinical use. Maintenance immunosuppression consisted of sirolimus combined with low-dose tacrolimus. This regimen, described initially at the University of Alberta, has been successfully replicated at other centers as part of a multicenter Immune Tolerance Network (ITN) trial [39,56]. More recently, induction with depletional therapies including thymoglobulin (6 mg/kg total dose) or alemtuzumab (30 mg intravenously) combined with the antiinflammatory agents etanercept (anti−tumor necrosis factor [TNF]) and anakinra (anti-IL receptor [ILR]) have been employed in the Edmonton program. We and others found that the more standard posttransplant combination of tacrolimus (6−10 ng/mL) and mycophenolate mofetil (up to 2 g/day in a divided dose as tolerated) is much better tolerated than sirolimus. The 3- and 5-year outcome data suggest more graft durability in terms of sustained insulin independence with this regimen, which has been encouraging.

In addition to the Edmonton Protocol immunosuppression described earlier, alternative regimens have been reported. The Minnesota Group, led by Dr. Bernhard Hering, used antithymocyte globulin (ATG) and etanercept (anti−TNF-α antibody) induction with a combination of sirolimus and mycophenolate mofetil with or without low-dose tacrolimus for maintenance, or hOKT3γ1 (Ala-Ala) (humanized anti-CD3 antibody) and sirolimus induction with sirolimus and reduced-dose tacrolimus for maintenance [47,48]. In some instances, alternative immunosuppressive agents have been used because of drug intolerance or other side effects. Islet patients often possess mild preexisting renal impairment as a result of long-standing diabetes, and this renal dysfunction may be exacerbated with calcineurin inhibitor therapy, even at the low doses involved in the Edmonton Protocol. The drug sirolimus may also have nephrotoxic side effects, which may be compounded when used in combination with a calcineurin inhibitor drug [57,58]. For these reasons, renal status must be monitored diligently in all patients after islet transplantation. In addition to its recognized nephrotoxicity, tacrolimus is associated with gastrointestinal side effects, which may lead to episodic diarrhea. Neurotoxicity may be seen with tacrolimus but is often avoided in

low-dose regimens [59]. Sirolimus is associated with neutropenia and mouth ulceration, but these side effects can be reduced with lower target trough levels and tablet formulations. In the context of islet transplantation, sirolimus has been linked to a number of side effects including dyslipidemia, small bowel ulceration, peripheral edema, and the development of ovarian cysts or menstrual cycle irregularities in female recipients [51,60]. Combined with the observation that sirolimus is extremely poorly tolerated at high doses in this patient population, this has led to avoidance of this drug within our program.

Although chronically immunosuppressed patients are at risk for developing all types of malignancy, squamous epithelial cancers most commonly occur and are most readily treatable. The Collaborative. Islet Transplant Registry reports an overall rate of less than 2% in islet transplant recipients [46]. The lifetime risk of lymphoma is estimated to be 1−2% in transplant recipients, but this risk is likely to be reduced in islet recipients, because these patients are generally not treated with glucocorticoids or OKT3.

FUTURE CHALLENGES

Overcoming Tissue Shortage

In its current form, islet transplantation is reserved for a small subset of patients with the most severe forms of T1DM. Even with the relatively small patient population selected for islet transplantation, the wait-list time for patients in Edmonton, which has access to organs from a large geographic region, ranges from 6 months to 2 years, depending on blood group. As islet transplantation becomes more suitable for a broader range of diabetic patients and as the incidence of diabetes increases, there will be an even more severe shortage of islet tissue for transplantation. Clinical islet programs rely on the scarce supply of pancreas organs derived exclusively from heart-beating, brain-dead cadavers. Compared with organs procured for whole-pancreas transplantation, which must fall within strict donor criteria, organs obtained for islet transplantation tend to be more "marginal" and come from older, less stable donors. Furthermore, the pancreas is particularly susceptible to toxicity from the circulating products of severe brain injury, hemodynamic instability, and inotropic support in a brain-dead organ or donation after cardiocirculatory death (DCD). The quality of the pancreas is further degraded by cold ischemic injury during transportation, which inevitably results in islet damage and loss. Brain death, with an acute cytokine and injury storm has been associated with islet injury, with consequential reduced islet recovery and viability compared to islet isolation in the absence of injury, at least in small animal models [61,62]. Because of the sporadic islet transplant experience with DCD pancreas donation in North America, a direct comparison with the standard neurological determination of death (NDD) donation has yet to be objectively conducted. A comparison study of clinical islet isolation success rates and transplantation outcomes between DCD and NDD pancreas donors from our center was published in 2015 [63]. Islet yields were similar between NDD and DCD. Likewise, metabolic function was similar between NDD and DCD, as well as the mean decrease in insulin requirement at 1 month after transplantation. Although these results support the broader use of DCD pancreata for islet isolation, a much larger DCD islet experience will be required to determine the noninferiority of both short- and long-term outcomes [63].

Unfortunately, once the pancreas is in the isolation laboratory, the extensive processing and purification steps during processing result in further islet destruction and loss, often resulting in at best 60% recovery of the estimated 10^7 IE/pancreas, [64]. As a result, nearly all islet recipients require islets derived from two cadaveric donors. Thus, a rapidly growing area of islet transplant research involves the development of improved cadaveric or alternative islet tissue sources for transplantation.

Living Donor Islet Transplantation

One approach to alleviating islet tissue demand would be to use living donors for islet transplantation. Living donor programs in kidney, liver, and lung transplantation have moved forward successfully at most leading transplant centers worldwide, in an attempt to meet the growing demand for donor organs and improve clinical outcomes. Long-term clinical outcomes of islet transplantation alone, in specialized centers, mirror the results of whole-pancreas transplant alone; over 50% of patients have achieved insulin independence 5 years after transplant [65]. However, apart from the University of Minnesota experiences [47] and those at the University of California, San Francisco (UCSF) [66,67], the rate of single donor islet transplant success remains low (10−15%) [68]. Therefore, most patients require multiple pancreas donors to achieve insulin independence. In addition, despite remarkable progress in clinical islet transplantation since 1999, islet supply and functional viability remain

significant challenges when islets are derived from cadaveric organ donors, even at the most experienced centers [61]. In the living donor setting, the distal half-pancreas could be procured under "ideal" circumstances without exposing the pancreas to hemodynamic instability or inotropic drugs, and the pancreas would be processed immediately without prolonged cold ischemia. Thus, the potency of islets derived from a living donor source is assumed to be far superior to cadaveric tissue. Living donor islet transplantation represents a unique opportunity to overcome donor organ shortage and procure the islet tissue under perfect conditions, with closer human leukocyte antigen matching between donor and recipient. Furthermore, the living donor islet transplant setting will provide a unique opportunity to develop protocols for pretransplant recipient conditioning for donor-specific tolerance induction.

Although cadaveric islet transplantation has been an active area of clinical research involving more than 1500 patients over several decades, only three cases of living donor islet allotransplantation have been reported [69–71]. Initially, the rationale of pursuing living donor islet transplantation originated from discouraging patient outcomes in the pioneering series of deceased donor islet allografts conducted in the late 1970s [72]; the first attempts at islet autografts (after pancreatectomy for chronic pancreatitis) proved to be more promising [71,73].

The first two clinical attempts at living donor islet allotransplantation were carried out in 1978 by Sutherland and colleagues at the University of Minnesota [70,71,73]. Although neither recipient achieved sustained islet function, these pioneering efforts were truly remarkable given the early stage of clinical islet transplant development at the time. The first recipient's graft was lost owing to a cytomegalovirus infection, and the second recipient lost function because of a sensitizing event as the result of a previously rejected kidney from the same donor (the recipient's sister) [72]. The immunosuppression available was primitive by current standards (azathioprine and high-dose steroids), and the islets were isolated using suboptimal conditions, before the development of the Ricordi chamber and sophisticated purification schemes currently used in clinical islet transplantation.

The dramatic improvement in clinical outcomes obtained in cadaveric islet transplantation since 2000 has renewed interest in developing living donor islet transplantation. The first living donor islet transplantation case attempted since the introduction of the Edmonton Protocol was carried out at the University of Kyoto in early 2005 [69]. The recipient, a 27-year-old woman, developed C peptide–negative, unstable diabetes after chronic pancreatitis as a child. Her 56-year-old mother was approved to be the donor, and islets were purified from the distal pancreas obtained during an open laparotomy. There were no surgical complications in either donor or recipient. The unpurified islet mass (408,114 IE [8200 IE/kg] in a volume of 9.5 mL after tissue digestion) was transplanted into the portal vein using the percutaneous approach under full systemic heparinization. Edmonton Protocol–style immunosuppression was started before transplant using sirolimus and low-dose tacrolimus (started 7 days before transplant), anti-IL2R antibody (given 4 days before transplant and on the day of transplant), and anti-TNF-α blockade induction (infliximab; given 1 day before transplant). Insulin therapy in the recipient was discontinued at 22 days after transplant; this patient continued to be insulin independent with excellent glycemic control and a normal HbA1C more than 1 year after transplant [74]. The donor presented no evidence of glucose intolerance, maintained normal HbA1C values, and was C-peptide positive 30 months after transplant [72,74].

Although no definitive conclusions can be drawn from this single successful case of living donor islet allotransplantation, results from living donor islet autotransplantation suggest that the insulin independence may be achieved routinely with significantly less IE/kg recipient body weight than has been required for cadaveric allografts thus far. It is widely accepted that over 70% of patients will remain insulin free after islet autotransplantation if an islet mass exceeding 300,000 IE ($\geq$2500 IE/kg) is transplanted, compared with the >10,000 IE/kg that is often required to achieve insulin independence with cadaveric islet preparations [75]. Reports from the Minnesota group showed that clinical islet autografts have a significantly lower rate of metabolic decay over time, even with a smaller islet implantation mass (<2500 IE/kg) [76]. The combination of single donor successes in islet allografts and the efficacy of a small islet mass required for insulin independence in islet autotransplantation have provided a stimulus for evaluation and potentially resurgences in living donor islet allograft programs [77].

Globally, living donors compromise a fraction of the pancreas–islet transplant (less than 1%); however, in countries with strict donor criteria and a severe donor shortage, such as Japan, living pancreas donors comprise over 20% of all organ donors [72,78]. Despite the potential risks for a living donor in terms of surgically induced diabetes and surgical complications [79], the demand for islet tissue, and the relative ease of implementing living donor protocols into established islet transplant programs, there is a likelihood that this approach may more forward, especially in countries with strict organ donor criteria.

Xenotransplantation

Living donor islet transplantation may circumvent the wait list for suitable donor tissue in some specialized cases; however, associated risks for the donor and the possibility of an insufficient islet yield to obtain insulin dependence remain significant concerns. Identification of a renewable and limitless xenogeneic source of islets would avoid the requirement for human islet donors altogether and could provide enough tissue to transplant diabetic patients as often as required. Pigs are particularly attractive as a xenogeneic islet source because they are widely available, produce insulin that is functional in humans, and could be selected for certain donor characteristics including genetic manipulation. For a few decades, it has been widely thought that of all of the experimental xenotransplantation strategies, islet transplantation is perhaps the closest to clinical application [80]. The first clinical case of islet xenotransplantation was conducted by Groth et al. in 1994, when fetal porcine islet cell clusters were placed under the kidney capsule [80]. Despite the lack of clinical benefit observed in this pioneering attempt of xenotransplantation, evidence that porcine islets could survive in the human body was demonstrated through the measurement of porcine C-peptide.

A limited small clinical experience was reported with porcine derived islets; however, few have resulted in reduced insulin requirements and no patients have achieved a period of insulin independence [81–83]. Despite these setbacks, islet xenotransplantation using porcine tissue has remained an active area of research, and progress has been made using preclinical nonhuman primate models implementing various genetic donor manipulation and immunosuppressive protocols [82,84–87]. Most notably, experimental studies have demonstrated that nonencapsulated pig islets (both wild-type and genetically engineered) have the capacity to maintain normoglycemia in immunosuppressed diabetic nonhuman primates for more than year after transplant [88,89].

The generation of α-1,3-galactosyltransferase–deficient pigs has provided a source of islet tissue lacking the major xenoantigens causing hyperacute rejection in pig-to-human xenotransplantation [90]. Still, it remains to be determined whether the transmission of endogenous retroviruses or other zoonotic infections from pig to human can be completely avoided in xenotransplantation, even with the establishment of highly monitored "clean" pig colonies [87,91,92].

The first nationally regulated clinical trial of intraperitoneal encapsulated (alginate-poly-L-ornithine-alginate) neonatal porcine islet xenotransplantation in nonimmunosuppressed diabetic patients was carried out in New Zealand and reported in 2014; it demonstrated a marginal reduction in hypoglycemic unawareness [93]. Curiously, the number of islets transplanted did not correlate to the clinical outcome, because the transplantation of 5000 IEq/kg was associated with better results than that of larger doses (15,000 and 20,000 IEq/kg) [88]. The authors speculated that the large islet mass was prone to oxygen and nutrient starvation, resulting in substantial islet loss.

Building on this first study, in 2016, Matsumoto et al. reported the results of a second nationally regulated clinical trial, conducted in Argentina, of encapsulated porcine islet xenotransplantation in nonimmunosuppressed patients with T1DM [94]. Differing from their initial clinical study, the islets were transplanted in two separate infusions 3 months apart, in the hope of reducing the hypoxia associated with large transplant volume. Patients received either 5000 IEq/kg × 2 or 10,000 IEq/kg × 2 of encapsulated neonatal porcine islets in the peritoneal cavity by laparoscopy (n = 4/group). Despite the demonstrated significant improvement in HbA1c and the reduction in hypoglycemic unawareness events for up to 2 years after transplant, the reduction in insulin dose was marginal [88,94]. There was no evidence of complications from the transfer of porcine endogenous retroviruses [95]. This second nationally regulated trial was indeed a progressive step forward for the field of clinical islet xenotransplantation; however, numerous aspects need consideration before large-scale clinical trials should be initiated [87].

Stem Cell Transplantation

Substantial research efforts have been made to identify suitable islet precursor cells that could be differentiated into a potentially unlimited source of insulin-producing β cells. Difficulties in producing physiologically regulated insulin secretion and control of proliferation have made progress in this area difficult to achieve histologically [96,97].

The pursuit of a renewable source of insulin-producing cells has led researchers to consider a multitude of tissue origins to derive these cells. The native pancreas contains progenitor cells capable of β-cell repopulation in the event of injury [98,99]. Given the proper environment and transcription factors, these cells can be directly reprogrammed into cells that closely resemble β cells [98,100]. Despite persuasive evidence that demonstrates the regenerative capacity the adult pancreas retains, it remains uncertain whether this ability is stem cell driven, because specialized cell types within or outside the pancreas indeed retain plasticity in proliferation and differentiation. As such,

inducing reprogramming or transdifferentiation of exocrine cells or other types of endocrine cells in the pancreas may provide a more efficacious approach to deriving insulin-producing cells.

Others have explored using hematopoietic stem cells as precursors to insulin-producing cells. This includes attempts to use bone marrow–derived cells in addition to umbilical cord blood (UCB). This was initially an exciting area of study, because UCB is easily obtained and would avoid some of the ethical implications associated with the use of stem cells. Unfortunately, early animal studies showed no conclusive evidence of endogenous β-cell replenishment after hematopoietic stem cell injection [101,102]. Even so, clinical studies have been conducted in type 1 diabetic patients. Haller et al. used stored autologous UCB infusions in newly diagnosed patients with T1DM, showing reduced insulin requirements and lower HbA1C [103]. A further study employing hematopoietic stem cells resulted in most of the 23 newly diagnosed patients with T1DM who received these cells achieving insulin independence and elevated C-peptide levels [104].

More recently, a prospective clinical study in which 20 adult patients with newly diagnosed T1DM were enrolled and randomized to mesenchymal stem cell (MSC) treatment or to the control group. One-year follow-up demonstrated residual β-cell function, as measured by serum C-peptide in response to a mixed-meal tolerance test, in the treatment patients in contrast to those in the control arm, who showed a loss of C-peptide. No side effects of MSC treatment were observed, which may constituted this procedure as a safe and promising strategy to intervene in disease progression and preserve β-cell function [105].

In addition, a study reported a single-center experience with suppressing T1DM progression by combining immunoablation and autologous hematopoietic stem cell transplantation (AHSCT) in newly diagnosed patients. During the 52-month median time of follow-up, 20 of 23 patients (87%) remained without the use of exogenous insulin for at least 9.5 months. Average HbA1C concentrations were 10.9% at diagnosis, 5.9% at 1 year, 6.4% at 2 years, 6.8% at 3 years, and 7.1% at 4 years after AHSCT. No severe complications of diabetes were seen; however, one patient died of pseudomonas sepsis in the course of neutropenia after AHSCT. Thus, AHSCT may lead to a remission of T1DM with good glycemic control in the vast majority of patients, with the period of remission lasting over 5 years in some [106]. The underlying mechanism of preserving endogenous β-cell mass using MSC has yet to be fully elucidated.

Owing to their pluripotency and ability to self-renew, embryonic stem cells (ESCs) have received an enormous amount of research attention. Since 2000, researchers have attempted to find the optimal set of conditions and signals to differentiate them into an insulin-producing cellular population. A number of the early attempts were conducted using rodent ESCs; although they were initially promising, they were limited by cell homogeneity, immaturity, the low number of insulin-positive cells, and a lack of glucose sensitivity [107–110]. It was not until 2004 and 2006 that an effective differentiation strategy was discovered, which paved the way for the differentiation of human ESCs (hESCs) into pancreatic endoderm cells (PECs) that contained both insulin and C-peptide [111,112]. Further refinement of the strategy allowed these cells to become glucose-sensitive, showing the ability to ameliorate diabetes in a rodent model [113]. Several alternative approaches have been developed to differentiate hESCs into pancreatic progenitor cells, which undertake the final stages of glucose-responsive β-cell differentiation in vivo [111,113–117]. Furthermore, advances in manufacturing PECs for clinical trials [118] and generating insulin-producing cells of increased maturity derived from hESCs or induced pluripotent stem cells (PSCs) in vitro have been reported with the capacity to restore normoglycemia rapidly compared with early progenitor cells [119,120].

In 2014, the US Food and Drug Administration and Health Canada granted ViaCyte Inc., a commercial leader in regenerative medicine technologies, permission to initiate the first-in-human pilot phase 1/2 clinical trial to test their VC-01 combination product in a small cohort of patients with T1DM. This product combines CyT49 hESC-derived PEC contained within a macroencapsulated immune-protective device transplanted subcutaneously (NCT02239354). It is hoped that early pilot results of this ongoing trial will the direct future progression of the field. Ongoing development of extrahepatic sites for stem cell–derived product implantation will likely require further optimization to improve engraftment, oxygen delivery, and metabolic exchange. However, it remains to be seen whether the transplantation of pancreatic progenitors in an immune-isolating device will be preferential to transplanting these or more mature glucose-responsive insulin-secreting cells into a retrievable prevascularized subcutaneous site in the presence of standard clinical immunosuppressive therapy.

In addition to ViaCyte, the commercial entities BetaLogics, and SemmaTherapeutics are continuing to perfect a protocol to differentiate PSCs into insulin-producing cells. ViaCyte's less differentiated PECs may prove longer to reverse diabetes compared with BetaLogics' and SemmaTherapeutics' more mature "β-like" cellular products; however, this time variable may not be the greatest concern for a patient with life-long T1DM, who may not mind waiting a few extra weeks to become insulin independent via stem cell differentiation.

Ethical and religious concerns regarding the use of ESCs remain, in addition to concerns pertaining to the risk of teratogenicity [121–123]. However, if a sustainable stem cell–derived, insulin-producing cellular product can be ethically derived from a donated, discarded human embryonic blastocyst from an in vitro fertilization clinic, a world supply of limitless β cells from one single discarded blastocyst could be achieved. Despite the remaining challenges, the clinical path for stem cell–derived therapeutic products has been paved, providing a tantalizing optimistic future for this β-cell replacement therapy to restore glycemic control to patients with insulin-dependent diabetes.

Optimal Transplantation Site

The intraportal infusion of islets in the liver has become the implantation site of choice, receiving more than 90% of clinical islet graft, and is the only islet microenvironment that routinely results in insulin independence. As the use of stem cell–derived β cells and xenogeneic islet sources becomes a clinical reality, an engraftment site that facilitates harmless retrieval may be compulsory. As a consequence, intensified research effort has been dedicated to pursuing alternative transplant sites [124–126]. It has been suggested that an optimal cellular transplantation site should (1) have an adequate tissue volume capacity; (2) be in close proximity to vascular networks; (3) have dynamic communication between the cellular graft and systemic circulation; (4) facilitate minimally invasive means to transplant, biopsy, and retrieve; and (5) elicit minimal inflammation [126].

The islet isolation process subjects islets to significant ischemic and physical injury, rendering them susceptible to posttransplantation stresses. Islets require ready access to oxygen and glucose and benefit from close proximity to a good vascular supply, because their revascularization is not immediate and their capacity for diffusion is limited. As an endocrine tissue, islets require a means to sample representative glucose levels and need to be able to deliver insulin through a relevant route to target tissues. Ideally, a transplanted islet should reside in a site with minimal immunological attack and low levels of posttransplantation β-cell apoptosis, such as that induced by the instant blood-mediated inflammatory reaction (IBMIR). From a surgical standpoint, it would be advantageous to have a transplant site that afforded minimal procedural complications and allowed one to monitor islets after implantation.

The portal vein–liver site has become the standard site in most islet transplants. An early rodent study showed this site to be superior with respect to the number of autologous islets required to reverse hyperglycemia [27]. However, further studies showed an eventual loss of islet function even in the absence of alloimmune or autoimmune attack [127]. Despite marked improvement in patient outcomes after intraportal islet infusion, the achievement of single-donor engraftment success has been challenging, because most recipients require at least two donors to achieve insulin independence [47,48]. The death of significant numbers of intraportal islets in the immediate posttransplant period from tissue factor–triggered platelet injury, the IBMIR, compounded by ischemia from immature revascularization contribute to impaired islet survival and function in the long term [128]. Various additional factors contribute to acute islet loss and graft attrition, particularly in the intraportal hepatic site, resulting in an estimated 70% loss of transplanted β-cell mass [129]. Finally, although islet infusion is relatively straightforward, there are possible complications, including bleeding and thrombosis. This site has indeed allowed islet transplantation to reach amazing clinical success, but there are clear reasons for a search to identify a clinically relevant, non–blood exposed extrahepatic site to optimize β-cell engraftment further.

Although the kidney subcapsular space has become the site of choice for many researchers employing a mouse islet transplant model, it has not shown promise in clinical practice. Major limitations of this strategy are surgical invasiveness, its systemic nature, nonphysiological glucoinsular response, hypoxia, and limited transplant volume capacity, coupled with differences in the renal capsule architecture between rodents and humans [130–133]. Although it is theoretically a tempting site, with its high oxygen content and proximity to endogenous islet location, the pancreas is relatively invasive to access and may potentiate the autoimmune attack of transplanted islets through the priming of local lymph nodes. Although the latter has not yet been proven, the usefulness of this site is nullified in the case of islet autotransplantation. Furthermore, limiting this site of islet implantation is the limited ability to retrieve and image, and the finite transplant volume capacity [134–136]. The formation of an omental pouch, created surgically using omentum and the parietal peritoneum, has shown efficacy in both rat [137] and dog [138] models of diabetes. Although necessitating a higher number of islets to reverse diabetes (compared with the renal subcapsular site), the omentum is a vascular, portal insulin delivery site and a possible location for implanting islet encapsulation devices [139–142]. A potential drawback to this site is the inability to retrieve and image and provoke an inflammatory response effectively. However, success in the first clinical patient at the University of Miami using BioHub technology has instilled a sense of renewed optimism for this site. A related structure in mice, the epididymal fat

pat, has been used successfully to transplant embryonic endocrine progenitor cells [113]. In addition, a pouch could be created laparoscopically, which minimizes the morbidity of surgery. Further research needs to be completed to determine the long-term survival of islets at this potentially useful site.

Researchers have shown that islets can be transplanted into the gastric submucosal space (GSMS) [143–146]. This site has many potential benefits including avoiding the IBMIR, a rich oxygen supply, and a high oxygen tension. In a preclinical animal study [143], it was shown that diabetic pigs receiving islets endoscopically into the GSMS fared better than did pigs receiving intraportal islets. Pigs in the former group showed less early islet loss and received less insulin to maintain normoglycemia. More recently, a technique of endoscopic-assisted biopsy of porcine islet allografts in this site demonstrated an additional benefit of the potential easy access for graft monitoring and biopsy [143–145]. This has become an exciting possibility for an extraportal site of islet graft deposition; further research should shed light on the potential for long-term graft survival at this site. Various immune privileged sites have also been investigated, including the brain [147–149], testis [150,151], thymus [152–155] and anterior chamber of the eye [156], which demonstrate promising preclinical results. However, the use of such a strategy to avoid chronic immunosuppression has yet to be translated to clinical islet transplantation.

In theory, subcutaneous transplantation should be superior to portal vein infusion because it provides ready access to the graft and the possibility of monitoring function through imaging [157–159]. However, transplantation of islets into an unmodified subcutaneous site has universally failed to reverse diabetes in animal models or in humans, owing to poor oxygen tension and inadequate vascularization [160]. Stimulation of angiogenesis is critical to successful subcutaneous islet transplantation [125,126,159,161]. Oxygen generators, polymers, meshes, encapsulation devices, matrices, growth factors, and cotransplantation of MSCs have all been explored with variable success.

Pepper et al. demonstrated the successful restoration of euglycemia using mouse and human islets in preclinical animal models, by using the subcutaneous deviceless transplant approach, which modifies subcutaneous tissue into a highly vascularized engraftment site by temporally exploiting the natural foreign body reaction to an implanted catheter [162]. This site was also efficacious in reversing diabetes after transplant in mice at a marginal islet dose [163]. This technical was further optimized and demonstrated the long-term durability of syngeneic islet grafts in mice [164]. The use of this transplant technique will be explored in a first-in-human trial planned at the University of Alberta.

Improving Engraftment Posttransplant

In clinical islet transplantation, islets derived from multiple donors are often required to achieve insulin independence, which suggests that a significant portion of the transplanted islets must fail to engraft and become functional. It has been estimated that up to 70% of the transplanted β-cell mass may be destroyed in the early posttransplant period [51,129]. Because this profound loss has been observed in both immunodeficient and syngeneic islet transplantation models, islet survival is likely regulated by non–immune mediated stimuli. After isolation, the islet microvasculature is completely disrupted, and upon implantation into the portal circulation, hypoxia persists whereas the islets revascularize, which can take up to 2 weeks [165–167]. During this engraftment period, the islets are continuously exposed to immunosuppressive drugs including tacrolimus and sirolimus, which are known to affect β-cell survival and function adversely [168,169]. These negative effects are likely compounded by the proximity of the transplanted islets and high concentrations of these drugs in the hepatoportal circulation, further degrading β-cell mass over time [170].

A critical process that may influence islet engraftment and survival in the early posttransplant period has been termed the IBMIR. Islets have been shown to express tissue factor naturally, a protein that acts as a receptor and cofactor for factor VII, an important mediator of the coagulation cascade [171]. Isolated human islets release tissue factor along with glucagon and insulin, which ultimately leads to platelet activation and binding at the surface of the islets. This causes the formation of a fibrin capsule around the islet and disruption of the islet morphology [128,129,171,172]. Most of this process has been characterized using an in vitro tubing loop model, so the true impact of this process in the clinical setting has yet to be fully characterized. However, examination of serum in patients undergoing islet transplantation has shown that a statistically significant increase in the serum concentration of thrombin–antithrombin complexes is present almost immediately after portal infusion, with peak levels occurring at 15 min, even when there was no clinical evidence of portal hypertension or intraportal thrombosis [171]. Because platelet activation is a primary contributing factor in the generation of an inflammatory response, IBMIR is probably an important early process in islet transplantation that elicits an immune response [128,129,171,172].

Many studies targeted at enhancing islet survival during the early posttransplant period have been published, and a variety of different strategies have been tested. Some groups aimed to enhance revascularization with vascular

endothelial growth factor, but these studies have not yet demonstrated that this approach significantly improves islet graft survival [173]. Anticoagulation strategies using injection of activated protein C or inhibition of thrombin have been studied as a means to inhibit IBMIR, but these interventions have shown only a modest benefit in a series of in vivo studies in animal models [172,174,175]. Anticoagulation with insulin−heparin peritransplant has aided in reducing the impact of IBMIR and increased single-donor islet engraftment success rates from 10% to 40% [176]. Clinical studies designed to prevent IBMIR, such as low−molecular weight dextran sulfate, are under investigation.

Because these processes described involve both extracellular (i.e., IBMIR) and intracellular (i.e., hypoxia) stimuli leading to β cell death, another approach to preserving β-cell mass in the early posttransplant period has been to directly inhibit the apoptotic triggers that ultimately lead to loss of islet mass after transplant. A variety of strategies have been explored in the experimental setting; although promising data have been generated in vitro, demonstration of in vivo benefit to islet graft survival has been more elusive [177−180]. Many studies have described inhibition of a variety of apoptosis-associated proteins, including cellular FLICE-inhibitory protein (prevents caspase-8 activation), A20 (inhibits nuclear factor-κB activation), Bcl-2, and Bcl-XL (mitochondria-associated antiapoptotic proteins) [177−182]. A20 has shown promise, because its overexpression reduced the islet mass required in syngeneic islet transplantation in mice [182,183]. Investigations using X-linked inhibitor of apoptosis protein, which inhibits the downstream effector caspases that function in the final common pathway of apoptosis, have demonstrated promise in both human and rodent models of engraftment and in promoting murine islet allograft survival [184−186]. However, this area of research is limited by its requirement for the genetic manipulation of islet tissue before transplant, which has proven to be variable and difficult to achieve in human islets. Also, these genetic alterations are most often regulated with viral vectors, which represent a highly controversial reagent for clinical use, especially in immunosuppressed transplant recipients.

Our group has investigated the use of small-molecule peptidyl pan caspase inhibitors to promote β cell survival during the posttransplant engraftment period. These data demonstrate that euglycemia can be achieved in more than 90% of transplant recipients after an 80−90% reduction in islet implant mass using mouse or human islets in a nonallogeneic transplant model in addition to porcine autografts [187−189].

Next-generation pan-caspase inhibitors, including EP1013 and IDN-6556, demonstrated augmented long-term engraftment using a marginal islet dose capable of restoring euglycemia effectively in transplant recipients in both small- and large-animal models [187]. We described the ability of the potent pan-caspase inhibitor F573 to reduce apoptosis effectively in murine and human islets. Furthermore, the authors demonstrated that F573 treatment could differentially augment islet engraftment in intraportal and extrahepatic transplant sites using full and marginal islet transplant doses in mice [190]. These data further suggest the therapeutic benefit of novel inhibitors in the clinical islet transplant setting. As such, the Edmonton group is investigating the use of next-generation pan-caspase inhibitor in their clinical islet program (NCT01653899).

Improved Immunomodulation: Toward Donor-Specific Tolerance

One unique component of islet transplantation in patients with T1DM is the possibility of recurrent autoimmunity, which may elevate the demand for immunosuppression. Indeed, it has been well-established using a rodent model of T1DM, the nonobese diabetic (NOD) mouse, that control of recurrent autoimmune reactivity to β cells is one of the most difficult obstacles to overcome in islet transplantation [191−193]. Although it has been challenging to study recurrent autoimmunity in clinical patients, some evidence exists suggesting that levels of autoantibodies to glutamic acid decarboxylase (GAD) and IA-2 increase after islet transplantation, although the direct impact of this phenomenon on graft survival has yet to be fully elucidated [194,195]. As such, translation of findings in animals to clinic has been challenging, as highlighted by a trial demonstrating that GAD65 immunization was ineffective in preventing islet destruction in patients with recent diabetes onset [196].

If recurrent autoimmunity alters immunosuppressive drug functional thresholds, this presents yet another problem in the context of islet transplantation, because many of the drugs are directly β cell toxic. Direct control of recurrent autoimmunity may enhance long-term graft function in islet transplantation. Attempts have been made to control autoimmunity at the time of diabetes onset using various immunosuppressive agents such as azathioprine, prednisone, cyclosporin A, or antithymocytic globulin, but no significant benefit was observed [197]. Clinical studies using a modified anti-CD3 (Ala-Ala) in patients with new-onset T1DM have demonstrated that this treatment significantly improved C-peptide responses in these patients, which persisted for up to 2 years after treatment [198]. More recently, the ITN reported the findings of the Autoimmunity-Blocking Antibody for Tolerance in Recently Diagnosed Type 1 Diabetes (AbATE) trial. This trail was the fourth with the monoclonal antibody teplizumab (anti-CD3) and demonstrated preservation of β cell function [199]. The AbATE trial demonstrated that in

responders, mean preservation of C-peptide continued at baseline levels for 2 years [200]. These results make a persuasive case for full-scale phase 3 trials designed with adequate doses to preserve C-peptide in newly diagnosed patients with T1DM [199].

Incorporation of this induction agent into clinical islet transplant protocols suggested that it may enhance insulin independence rates after single-donor infusion, which may be related to its ability to curtail β-cell autoimmunity in these patients [48]. Continued development of therapies targeted at regulation of autoimmunity will allow further refinement of immunosuppression protocols for islet transplantation in the future.

In all types of transplantation, the ultimate goal is to develop therapeutic protocols that involve a brief period of treatment only during the initial posttransplant period, followed by the complete withdrawal of all immunosuppressive drugs. This phenomenon has been termed "operational tolerance," because it may involve passive ignorance of the graft or a more active T-cell tolerance to the graft antigens. In experimental transplantation, the difference in these two types of response is important and can be measured using retransplantation of donor-type or third-party tissue, with tolerance resulting in acceptance of the donor-type graft and rejection of the third-party graft. In the clinical setting, however, the distinction may not be as critical, because both ignorance and tolerance would allow for reduction or withdrawal of immunosuppressive therapies. The most widely studied pathway to tolerance induction involves the inhibition of T-cell costimulation after T-cell receptor ligation. During an immune response, a T cell must receive "signal 2" through interactions between its surface molecule CD28 and CD80 or CD86 on the antigen-presenting cell to become fully activated. To disrupt this interaction, the extracellular portion of CTLA-4, which has a higher affinity for CD80/CD86 than CD28, has been artificially fused with human Fcγ to produce the soluble molecule CTLA4-Ig, designed for therapeutic purposes. CTLA4-Ig has been recognized for its potent immunoregulatory activity in murine models of T1DM, in which treatment of young NOD mice dramatically reduced the incidence of T1DM [201]. Our program and others demonstrated that CTLA4-Ig treatment in allogeneic islet transplantation can prolong graft survival but does not induce tolerance [202–204]. A high-affinity version of CTLA4-Ig called belatacept or LEA29Y has been developed for clinical use and has shown considerable promise in promoting allograft survival in nonhuman primates and in clinical renal transplantation [205,206]. Two compounds have been approved for use: abatacept (CTLA4Ig) and belatacept (LEA29Y), which block T-cell interaction with CD80/CD86 on antigen-presenting cells. The group at UCSF reported promising results with belatacept costimulation blockade combined with thymoglobulin (thymo)-based therapy [66]. This protocol allowed a calcineurin inhibitor–free maintenance therapy strategy and even permitted single-donor engraftment success. This same group studied the benefit of an anti-LFA-1 antibody called efalizumab [67]. Although effective in allowing tacrolimus-free maintenance therapy, it was withdrawn from clinical use owing to concerns regarding progressive multifocal leukoencephalopathy, and thus impeded the long-term follow-up.

A second costimulatory pathway that was examined in transplantation involves the interaction between CD40 on antigen-presenting cells and CD40L (CD154) on T cells, leading to T-cell activation. This interaction also promotes B-cell differentiation and the activation of antigen-presenting cells including macrophages and dendritic cells. Blockade of this pathway using anti-CD154 therapies demonstrated considerable promise in promoting tolerance induction in primate models early on, but further testing of the potent anti-CD154 blocking antibody (Hu5C8) was halted owing to unexpected thromboembolic complications in clinical trials [207–209]. The development of therapeutic antibodies targeting the CD40 molecule appears to avoid this negative side effect and should prove to be important in future clinical tolerance induction protocols in islet transplantation [210].

The combination of multiple immunotherapy approaches may prove to be most efficacious in achieving desirable, durable, long-term transplant outcomes. Blockade of IL-1 with anakinra, an IL-1 receptor antagonist, has become important in treating inflammatory conditions including rheumatoid arthritis [211]. In clinical islet transplantation, improved efficacy was demonstrated when combined iodixanol-based islet purification, thymoglobulin induction, and blockage of IL-1β and TNF-α was implemented [212]. Our own laboratory investigated the combination of TNFα and IL-1 blockade and found them to be synergistic in promoting islet engraftment in mice [213].

Bellin et al. reported a comprehensive study in which patients receiving anti-CD3 monoclonal antibody alone or ATG or alemtuzumab plus TNF-α inhibition exhibited substantial long-term insulin independence compared with patients treated with anti-IL-2R [214]. Furthermore, these outcomes were comparable to that seen with pancreas transplant alone. Findings support the potential for long-term insulin independence after transplant using potent induction therapy, with anti-CD3 Ab or T-cell depletion with TNF-α blockage [214].

SUMMARY AND CONCLUSIONS

β-cell replacement through islet transplantation presents the best opportunity to treat T1DM and prevent long-term serious complications associated with this disease. The concept of islet transplantation is not new, but investigators struggled to find success in achieving insulin independence until the introduction of the Edmonton Protocol in 2000. This has provided hope for many patients with diabetes, but in its current form, islet transplantation is reserved only for patients with the most severe disease. Restoration of endogenous-regulated insulin by the liver through intraportal islet transplantation has been highly effective in mitigating hypoglycemic reactions, and current data from Edmonton indicate that almost 70% of patients continue to secrete measurable C-peptide by 18 years after transplant, which greatly improves the quality of life for many patients [45]. Although up to 80% of recipients may attain insulin independence at 1 year after transplant, insulin independence has tended to wane over time. Protocols from the leading centers suggest that insulin independence may be maintained in up to 50% of patients by 5 years after transplant, but Collaborative Transplant Registry data collected across a larger number of participating centers still suggests that insulin independence is maintained in a much lower proportion over time.

However, the requirement for islets derived from two or more cadaveric donors severely limits the availability of this procedure. There are multiple opportunities for intervention throughout the entire process, from pancreas procurement, shipment, and islet processing to strategies for enhanced islet survival after implantation. Priority areas for clinical trials include the expansion of living donor protocols, interventions to impede the IBMIR process, and the use of nondiabetogenic and more "islet-friendly" immunosuppressive and tolerance-induction strategies to control both autoimmunity and alloimmunity effectively. Strategies targeted at preserving β-cell mass throughout the process will have a substantial and immediate impact on islet transplantation by reducing the amount of islet tissue necessary to reverse diabetes. Once some of these obstacles are overcome, islet transplantation will become available to a broader population of patients with T1DM, especially those early in the progression of the disease who will benefit most because the development of serious chronic secondary complications could be avoided.

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CHAPTER

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Prenatal Cell- and Gene-Based Therapies for Regenerative Medicine

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INTRODUCTION

Regenerative medicine promises the restoration of structure and/or function of deficient or damaged tissues and organs, with the goal of finding a way to cure previously untreatable injuries and diseases [1].

When contemplating the use of regenerative medicine during the prenatal period, one must first consider which diseases could benefit the most from such treatment. For example, in inherited metabolic diseases such as Lesch–Nyhan, Tay Sachs, and the acute neuropathic forms of Gaucher, a considerable amount of irreversible neuronal damage occurs during embryonic and fetal development [2]. The damage that these diseases exert during gestation cannot be entirely reversed after birth, causing even the most advanced treatments given to these patients postnatally to fall short of full therapeutic benefit [3]. Hence, the most compelling rationale for using regenerative medicine approaches during gestation is the ability to treat diseases early enough in development to prevent the onset of structural or metabolic damage, and thereby avoid the devastating manifestations that would otherwise occur before birth. Even in patients with diseases that can be cured postnatally, a persuasive argument can be made for the psychological and financial benefits of performing correction in utero, because it would allow the birth of a healthy infant who ideally would require no further treatments.

Remarkable advances in prenatal imaging, such as high-resolution ultrasonography and high-throughput molecular techniques, have improved the ability to identify diseases early in gestation by using fetal cells, or cell-free fetal DNA present in the maternal blood [4], the latter of which essentially eliminates any risk to the fetus during diagnosis. Importantly, these technical advances have not only improved the ability to identify diseases early in development, they have made it possible to deliver therapies safely to precise anatomic sites within the early-gestation fetus. Therefore, preemptive fetal regenerative medicine would entirely transform the current treatment of genetic disorders [5], allowing physicians to intervene before clinical manifestations of disease. It is also worth mentioning that the environment within the uterus provides a sterile platform in which to manipulate a fetal recipient. For instance, if one considers the treatment of an immunodeficiency, the maternal womb functions as a sterile incubator, allowing immune reconstitution before exposure to pathogens [5].

In this chapter, we review the therapeutic rationale for using in utero stem cell transplantation (IUTx), and/or in utero gene therapy (IUGT) in the context of regenerative medicine and examine crucial experimental data to support their use. We discuss the fetus as a target for regenerative medicine and the biological barriers that have thus far precluded more widespread clinical application/success of prenatal regenerative medicine, and we illustrate some of the unique advantages the fetal patient possesses, as well as some of the potential risks that will need to be addressed before clinical implementation.

FETAL DEVELOPMENT AND REGENERATIVE MEDICINE

There are several biological advantages unique to fetal development that provide compelling evidence to believe that for certain genetic disorders, regenerative medicine approaches such as stem cell transplantation and/or gene

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therapy would be far more efficient and effective if administered during fetal life rather than postnatally. Because hematopoietic stem cells (HSC) were the first cell type tested for IUTx and they are the cell type that has been used in most IUTx studies, this chapter will focus on the use of HSC in IUTx. However, other cell types have also been explored in the context of IUTx. The choice to use HSC for IUTx was based on the ability of HSC to self-renew and differentiate into all of the mature hematopoietic lineages, which make them well-suited for treating a broad range of hematopoietic disorders. Equally important, their property of self-renewal allows successful HSC transplantation to result in lifelong correction. HSC have been the subject of intense study for decades and are the most comprehensively characterized stem cells in the body. They are one of the only stem cells in the body that has been isolated to relative purity, and much of what we know about the biology and behavior of stem cells in general is based on the paradigm that experiments with HSC established.

The initial rationale for performing HSC IUTx was based on the expectation that normal developmental events such as (1) the lack of a fully functional immune system, (2) migration of HSC from the liver to the marrow, and (3) the development of new hematopoietic niches occurring during the emergence of the hematopoietic/immune system would collectively create the proper setting to facilitate engraftment of allogeneic cells, thereby avoiding the complications and toxic myeloablative conditioning associated with postnatal bone marrow (BM) transplantation [6].

Definitive hematopoiesis commences in the yolk sac and/or aorta−gonadal−mesonephric region, migrates to the fetal liver, and finally transfers to the BM, where it resides postnatally for the remainder of life [7]. In addition, during fetal development, large-scale migration of stem cells occurs to seed tissue compartments [8]. It was therefore expected that the normal fetal biology would allow transplanted cells to piggyback onto the naturally occurring processes of migration, engraftment, differentiation, and expansion. Looking specifically at hematological disorders, it was presumed that donor reconstitution of the defective hematopoietic compartment would occur without the need for cytotoxic myeloablation, which is a primary cause of the severe morbidity and mortality associated with postnatal BM transplantation. Unfortunately, it is now recognized that the fetal hematopoietic system is highly competitive and represents a daunting barrier to engraftment of transplanted adult HSC [9−11]. However, if postnatally derived HSC possessing proliferative capability similar to that of fetal sources were able to be identified, or the regulatory signals controlling the migration of HSC and their seeding of nascent marrow niches were better understood, it is likely that these processes could ultimately be manipulated to drive the engraftment of donor cells [12].

A distinct advantage of delivering either cellular or genetic therapies during the fetal period, over a pediatric or adult patient, is the very small size of the fetus. At 12 weeks of gestation, the point in development at which prenatal therapy would commence, the human fetus only weighs roughly 35 g [4]. Therefore, it is possible to administer much higher cell or vector doses on a per-kilogram basis than could be achieved after birth. The ability to achieve much higher vector-to-target cell ratios should greatly enhance the efficiency of transduction. In addition, the ability to administer a small volume of vector and achieve the desired rate of transduction is of additional benefit from a technical and logistical standpoint, because the large-scale production of certain vectors under good manufacturing practice conditions is often extremely difficult.

Additional aspects of the developing fetus that make it a more suitable gene therapy recipient than the adult are the highly proliferative state of the stem or progenitor cells present throughout the body and the accessibility of these cells. γ-retroviruses and lentiviruses have received a great deal of attention as gene delivery vectors, owing to their ability to integrate into the genome of the host cell. This unique property allows transduction of a long-lived cell to provide lifelong therapy. In the adult, most stem cell populations are relatively quiescent, and they are often inaccessible, because of their tissue distribution and/or the presence of anatomic barriers. As a result, these promising vectors have often yielded fairly low levels of initial transduction and only a limited degree of expansion of transduced adult cells occurs after gene therapy, which have often prevented therapeutic levels of gene correction. During specific developmental periods, however, stem and progenitor cell populations are present at a relatively high frequency in most tissues and may be accessible to gene transfer, providing a unique window of opportunity for gene transfer to these expanding nascent stem cell populations, which will be inaccessible later in life [6,13]. In the fetus, the cells in all of the organs are actively cycling to support the continuous expansion that occurs throughout gestation. Thus, most cell types that are largely quiescent in the adult are far more mitotically active in the fetus. As such, these cells should be far more amenable to genetic correction with vectors requiring cell division. Although the active cell cycling in the fetus enables efficient transduction with vectors that require mitosis, this ongoing proliferation in all of the fetal organs is also beneficial when using vectors that do not have an absolute requirement for mitosis. The active cycling of the cells in all of the organs to support the continuous expansion that occurs throughout gestation should result in expansion of the gene-corrected cells during the remainder of gestation,

allowing initial transduction of even small numbers of target cells to result in significant levels of gene correction by birth. Clearly, being able to take full advantage of this fetal expansion will likely require the use of vectors that integrate into the host genome, because vectors based on nonintegrating viruses, such as adenovirus, or those that integrate only rarely, such as adeno-associated virus (AAV), will largely be lost during cellular division, causing cessation of a therapeutic effect.

In addition to the ability to access nascent stem cell populations, fetal biology provides one of the most compelling reasons to deliver cellular or gene therapies in utero: the possibility of inducing donor-specific immune tolerance [14]. Early in gestation, the emerging immune system is shaped by a process of self-education. In the fetal thymus, prelymphocytes that recognize "self"—major histocompatibility complex (MHC) are preselected, and any of those that exhibit the ability to recognize, with high-affinity, any of the myriad self-antigens in association with self-MHC, undergo deletion [15]. This process thus strives to create an immune system devoid of self-reactive lymphocytes (which could later lead to autoimmunity) and is populated with a diverse repertoire of lymphocytes that are able to recognize foreign antigens in association with self-MHC [6,16]. Therefore, the introduction of allogeneic cells or foreign proteins by IUTx, with subsequent presentation of exogenous antigens in the thymus, before the completion of thymic education, should lead to the deletion of alloreactive T cells, creating donor-specific or protein-specific immune tolerance.

Looking specifically at IUGT, the immature immune system of the fetus should enable the delivery of immunogenic transgenes or viral vectors that would be rejected by the intact immune system of a postnatal patient. For example, most of the world's population has been exposed to, and harbors antibodies to, the capsid proteins in various serotypes of AAV, which makes postnatal gene delivery with these valuable vectors difficult. Intervening in utero, before maturation of the immune system, would likely allow efficient gene transfer with these vectors, because these antibodies are not present. With respect to the transgene, many patients have the genetic diseases that are targeted with postnatal gene therapy because they do not produce a specific protein. As a result, their immune system has never "seen" this protein, and, after gene therapy, the cells of the immune system seek to eliminate any cells in the body that are expressing the very protein that could cure the patient of his or her disease. The low levels of gene delivery to the desired target cells and the immune response combine to yield low levels of expression of the therapeutic protein, and even these small amounts are often produced only for a short time. Performing IUGT could induce a state of tolerance to the transgene and perhaps to the vector itself, which not only ensures long-term, stable transduction and expression but should make it possible to administer postnatal "booster" treatments (if required) with the same vector and transgene without eliciting an immune response. An important caveat to inducing tolerance to the vector, however, is that such tolerance could potentially render the individual susceptible to postnatal infection with the wild-type virus on which the vector was based. Such an outcome is clearly undesirable; preclinical animal studies will be needed to determine whether this risk exists.

PRECLINICAL ANIMAL STUDIES OF IN UTERO STEM CELL TRANSPLANTATION

In 1945, Owen [17] described for the first time that the shared placental circulation present in monochorionic dizygotic cattle enabled an intrauterine exchange of circulating HSC that resulted in lifelong hematopoietic chimerism and donor-specific tolerance to the twin [17—19]; reports of natural chimerism in both nonhuman primate and human twins followed years later [20,21]. The presence of hematopoietic chimerism led to a lack of alloreactivity between the two siblings, and in the case of dizygotic human twins, the frequency of chimerism was relatively high (8% for twins and 21% for triplets) [22,23]. Because these levels of chimerism would be sufficient to exert a therapeutic effect in most hematologic diseases [6], these findings provided compelling evidence that allogeneic hematopoietic cells could engraft and induce durable immune tolerance if introduced early during fetal development; they inspired scientists decades later to consider using IUTx to treat hematopoietic disorders.

Although these "experiments of nature" hinted at the therapeutic potential of IUTx, these natural chimeras result from placental vascular anastomoses. As such, there is a continuous exchange of blood and contact with allogeneic cells and antigens beginning very early and persisting throughout gestation [24]. Unfortunately, it would likely be impossible to recreate this process experimentally, or ultimately, in a clinical setting to treat human patients. With this caveat in mind, however, these exciting findings provide the scientific basis for the therapeutic promise of IUTx, and for its ability to induce donor-specific immune tolerance, and they have served as the impetus to realize IUTx's clinical potential.

These observations in dizygotic twins are important because they provide compelling evidence that the immunological tolerance induced by IUTx can facilitate maintenance of the donor hematopoietic cells. Perhaps even more

exciting has been the realization that even if the levels of donor cell engraftment after IUTx are insufficient to be curative or therapeutic, one could exploit the antigen-specific tolerance induced by IUTx and administer postnatal "booster" transplants of same-donor HSC to achieve therapeutic engraftment without having to employ toxic myeloablation [25–27], or possibly even transplant same-donor solid organs.

With respect to using IUTx to induce donor-specific tolerance to permit subsequent transplantation of a same-donor organ, Billingham and colleagues performed groundbreaking experiments in the 1950s [14] that provided the first experimental evidence that IUTx with allogeneic cells could indeed induce donor-specific tolerance that permitted successful postnatal grafting of skin in mice. Given the robust nature of the immune response that is formed to third-party skin [28], this was a remarkable achievement and provided unequivocal proof of the durability of the induced state of tolerance. However, despite the demonstrated potential for in utero hematopoietic chimerism to induce immune tolerance, it was not until nearly 20 years later that Fleischman and Mintz began to explore the possibility of performing experimental IUTx with the goal of engrafting donor (allogeneic) HSC. Using a line of c-kit–deficient mice with genetic anemia, these innovative investigators showed that performing IUTx with allogeneic adult BM HSC reversed genetic anemia [29]. These important studies revealed another interesting aspect of donor cell engraftment after IUTx: the extent to which the donor cells repopulated the erythroid compartment of the recipient was directly correlated to the degree of anemia present. As a result, the erythroid compartment of lethally anemic homozygous mice was rapidly and completely replaced with donor-derived erythropoiesis, whereas donor-derived erythropoiesis was far less in mice with mild anemia. Mintz continued his work with IUTx, and ultimately made the striking discovery that erythroid reconstitution could be achieved in this model following IUTx with just a single HSC [30].

These early studies were predominantly performed with the goal of gaining a better understanding of HSC biology rather than trying to develop IUTx as a potential therapy. Nevertheless, these studies provided the first evidence that host cell competition could act as a barrier to donor cell engraftment after IUTx, a realization that would ultimately prove critical to successful clinical implementation of IUTx. It was almost 20 years from Mintz's initial report before independent confirmation came for the ability to achieve robust hematopoietic chimerism after IUTx in stem cell–deficient recipients [31], when Bruce Blazar demonstrated that only cells of the lymphoid lineage were reconstituted when mice with a T-cell defect were treated by IUTx [32,33]. These studies reaffirmed the concept that in the presence of a lineage deficiency, IUTx is able to reconstitute the defective lineage, but donor cells contribute minimally, if at all, to other hematopoietic lineages. These findings thus suggested that achieving clinically meaningful levels of donor cell engraftment after IUTx in recipients with a competitive hematopoietic compartment was likely going to be far more difficult than initially appreciated. Indeed, early IUTx studies in immunocompetent, wild-type mice consistently reported very low engraftment rates, and it was soon reported that immunodeficient mice engrafted much more efficiently than wild-type [33,34].

Since these initial pioneering murine studies nearly a half century ago, the field of IUTx has made substantial advances, moving first to large-animal models and ultimately to human trials. The first report of the engraftment of allogeneic cells after fetal transplantation in a large-animal model was made in sheep [35]. Among the various animal models for studying IUTx, sheep have proven to be particularly valuable as a preclinical tool. Fetal sheep provide a natural, unperturbed environment in which to study IUTx. Moreover, if IUTx is performed early enough in gestation, xenogeneic cells are not rejected, which makes it possible to study the engraftment and differentiation capacity of a variety of human stem cells [36]. Although it may not be readily apparent, sheep actually share many important physiological and developmental characteristics with humans. Importantly, the results of extensive studies of mammalian development and fetal physiology obtained in the sheep model have been directly applicable to an understanding of human fetal growth and development [37]. In contrast to most large animals, e.g., dogs and pigs, that tend to have large litters, sheep, like humans, typically have only one or two offspring in each pregnancy. In addition, sheep are similar in size and weight to humans during gestation, at birth, and even as adults. This makes it possible to develop and test clinically relevant doses of cells and vectors in this model, and then to translate the outcome directly to the clinic without the need for costly and often complicated scale-up. In addition, their immune system and hematopoietic development during fetal ontogeny have been thoroughly delineated and parallel those of humans [38–41], which make this model ideal for investigating the immune facets of IUTx (and IUGT). Also, sheep in captivity normally live for 8–10 years. This long life span allows important issues such as long-term efficacy and safety to be carefully evaluated.

The fetal sheep model has also had a critical role in defining the phenotype of long-term engrafting human HSC and was responsible for identifying several novel human stem cell markers/phenotypes, such as $CD34^{+}CD38^{-}$, $HLA\text{-}DR^{-}$, $Thy\text{-}1^{+}$, CD133, KDR, and $CD34^{-}$ [42–46], which underscores the high translational value of data obtained with this model. The sheep model has made it possible to define the role of the marrow microenvironment

in the engraftment of HSC after IUTx [37,47–49] and has also proven to be a valuable preclinical model in which to study HSC mobilization; engrafted human hematopoietic cells respond to human cytokines similarly to their native counterparts [50]. These data collectively support the utility of the fetal sheep model to develop and test IUTx (and IUGT) approaches and obtain clinically relevant results.

Like sheep, the canine model has been shown to accept xenogeneic transplants, and low-level multilineage hematopoietic engraftment has been demonstrated in hematologically normal dogs [27]. More recently, Flake and colleagues showed that the levels of hematopoietic engraftment in canine recipients after IUTx are sufficient to (1) ameliorate or cure the clinical phenotype of the canine analog of human leukocyte adhesion deficiency (canine leukocyte adhesion deficiency), and (2) induce donor-specific tolerance in some animals that is adequate to facilitate postnatal "boosting" of chimerism using a low-dose, busulfan-conditioning regimen, followed by transplantation of same donor T cell–depleted BM [27,51–53]. In addition to sheep and dogs, IUTx has been performed in pigs [54] and goats [55,56], and low-level engraftment has also been achieved after IUTx in nonhuman primates [57–60]. Subsequent studies in the pig model provided compelling evidence that IUTx with adult BM-derived HSC induced immune tolerance that enabled postnatal transplantation of a solid organ (kidney) allograft [61]. These exciting studies suggest that one day it may be possible to use IUTx to induce immune tolerance in fetuses with congenital abnormalities that require postnatal organ transplantation.

Despite the unquestionable clinical value of large-animal models, the low cost, simplicity of handling and housing, and ease of genetic manipulation have led investigators to use mouse models extensively to probe various components of the immune system to understand the mechanism(s) better by which IUTx induces immune tolerance. Flake, MacKenzie, Peranteau, Shaaban, Nijagal, and colleagues have performed elegant studies that collectively have provided compelling evidence that clonal deletion, anergy, and induction of donor-specific T regulatory cells (Tregs) all have important roles in establishing chimerism and inducing tolerance after IUTx [62–65]. These murine studies also revealed that even very low levels of allogeneic HSC (1–2% engraftment) are often sufficient to induce postnatal tolerance successfully across full MHC barriers, as long as a certain "threshold" level of chimerism is achieved and maintained [66]. Subsequent work by Shaaban et al. provided evidence that this engraftment threshold is likely governed by natural killer (NK) cell tolerance [67].

Collectively, these results in small and large animals have clearly demonstrated the technical feasibility of IUTx, validated its ability to induce donor-specific tolerance, and clarified some of the pathways that are required for tolerance induction. Although it is possible that the human immune system will present its own unique challenges, these animal studies establish an essential foundation for the ultimate clinical application of IUTx.

Barriers to In Utero Stem Cell Transplantation Success

One of the driving assumptions behind IUTx was that a so-called "window of opportunity" existed before the appearance of mature T cells in the fetal thymus and peripheral circulation (~12–14 weeks of gestation in the human fetus) [16] during which the fetus should be "preimmune" [68]. However, accumulating data from numerous experimental studies made it increasingly clear that significant barriers existed that prevented successful engraftment after IUTx unless the recipient possessed a lineage-specific defect, e.g., anemia or severe combined immunodeficiency (SCID), that could confer a competitive advantage to the donor cells [68–73]. It was the surprising findings of Peranteau [74], however, that conclusively dispelled the belief that the fetus is truly "preimmune," by showing (in mice) that IUTx with congenic cells produced more consistent engraftment and at higher levels compared with phenotypically identical allogeneic cells. These studies provided some of the first concrete evidence that the fetal immune system poses one of the major barriers to engraftment after IUTx. Further analysis of these congenic and allogeneic IUTx recipients revealed that although 100% of allogeneic and congenic recipients maintained high levels of engraftment up to 3 weeks after IUTx, 70% of allogeneic animals lost their engraftment between 3 and 5 weeks whereas congenic animals all remained chimeric [74].

The authors confirmed the presence of an adaptive cellular and humoral alloresponse that was quantitatively higher in nonchimeric versus chimeric animals, which placed these results at odds with a wealth of prior data, much of which was from this same group, and demonstrated long-term chimerism in a percentage of recipients (both mice and other animals) after IUTx and the presence of deletional tolerance. A possible explanation for this seeming discrepancy came from the authors' subsequent observation that allo-IUTx pups placed with surrogate mothers maintained their chimerism in the long term, and they became tolerant to the donor via a mechanism of partial deletion of donor-reactive T cells and the induction of a potent Treg response [63,75]. These findings suggested that IUTx can trigger maternal alloimmunization and that subsequent transfer of alloantibodies to the pup via breast milk resulted in the loss of chimerism.

In an independent, contemporaneous series of murine IUTx studies, Nijagal, MacKenzie, and colleagues provided further evidence that fetal intervention can trigger maternal alloimmunization. However, data from these studies led the authors to conclude that the trafficking of maternal T cells to the fetus was responsible for the loss of chimerism rather than alloantibodies in the breast milk [64,76]. Of course, one must recognize that placentation, maternofetal antibody−cell trafficking, and the time course of events after IUTx are likely to differ considerably between mice and large animals or humans. Nevertheless, these findings raise the important question of whether maternal immunization can limit engraftment after IUTx, and it may be an issue that needs to be considered when applying IUTx clinically. Until this question is addressed in clinically predictive large-animal models, the prevailing consensus in the field is that it may be prudent to use maternal cells in any clinical application of IUTx to eliminate the possibility of triggering a maternal immune response.

Although the preceding studies were well-designed and the data appear to be beyond reproach, Shaaban and colleagues made the astute observation that the maternal immune system has been intact in all human patients who have thus far undergone IUTx for a variety of diseases, despite the nature of the clinical outcome (success or failure). This group logically contends that these clinical data argue strongly against maternal immunity having an important role in engraftment failure after IUTx [67,77]. This group spent several years investigating the ontogeny of the fetal immune system in an effort to establish which cells or pathways present at the time of IUTx could account for the apparent immune barrier to engraftment of allogeneic cells. These authors identified a subset of early alloreactive NK cells within the fetal liver, which they thought might pose a barrier to engraftment of transplanted cells as early as the end of the first trimester in humans. This conclusion is supported by subsequent work from the same group, showing that it is possible to achieve engraftment of allogeneic cells reliably and consistently after IUTx if NK cells are depleted from the fetus, but not from the mother [67,77,78]. Intriguingly, the authors also found that the levels of early chimerism required to induce NK cell tolerance agree precisely with the threshold levels of engraftment that were discussed earlier. In further work, this same group established a possible mechanistic link between the induction of prenatal NK cell tolerance and the process of trogocytosis, which explains how levels of engraftment of only 1−2% could result in the exposure of donor antigens to a sufficient number of NK cells to induce donor-specific tolerance reliably [79]. Although these important findings have not yet been reproduced in large-animal IUTx models, these sophisticated studies collectively argue persuasively for the import role fetal NK cells have in achieving engraftment of allogeneic cells after IUTx, as occurs in postnatal HSC transplantation.

In addition to the possible immune response of the fetus and/or mother to the donor cells, host cell competition presents a significant barrier to engraftment of allogeneic HSC after IUTx. In postnatal HSC transplantation, the recipient is myeloablated before infusion of the donor cells to suppress endogenous hematopoiesis and create space within the BM niches for the infused cells. After IUTx, the situation is drastically different, however; the infused donor cells must compete against the robust endogenous fetal hematopoietic system. Studies in c-kit−deficient mice have lent strong support to the idea that donor (adult) cells may have a competitive disadvantage in the fetal environment by showing that in the absence of host competition, as few as one or two normal HSC can fully reconstitute the hematopoietic compartment after IUTx [29]. Similarly, IUTx studies performed in SCID mice illustrate the importance of host cell competition [32], in that donor cells completely repopulate the defective lymphoid lineage of the recipient. However, when no defect is present, the scale tips dramatically in favor of the endogenous fetal HSC, which have a marked competitive advantage over their adult-derived counterparts [9] owing to their accelerated or enhanced cycling and expansion kinetics. Data from the congenic mouse model of IUTx provide a striking example of how profoundly this competition from endogenous hematopoiesis can limit long-term donor cell engraftment. In this setting in which no immune barriers exist, even when investigators delivered massive doses of 2×10^{11} donor cells/kg, levels of long-term donor cell engraftment never exceeded 10% [74].

The limited number of available niches and the proliferative capacity of the fetal environment have also been suggested to serve as a barrier to successful IUTx [80]. For donor cells to engraft successfully, they must be able to compete favorably with the host cells for available hematopoietic niches. Support for this concept was provided by studies in which more competitive fetal donor cells or larger doses of adult cells were shown to enhance the success of IUTx [12,81]. Selective depletion of host HSC before BM transplant resulted in high rates of engraftment in adults, so it stands to reason that improved competition for available host niches after IUTx should also lead to higher levels of early chimerism. However, no evidence was found to suggest that quantitative or qualitative differences exist in the number of HSC or available niches in patients with SCID compared with patients with the range of other disorders that have proven refractory to correction by IUTx [82,83]. The absence of such data makes it hard to explain the marked difference in donor cell engraftment after IUTx in immunodeficient versus nonimmunodeficient recipients by invoking a competitive niche model [67]. Nevertheless, it is reasonable to assume that the fetal marrow indeed possesses a finite number of hematopoietic niches that are available for donor cells after IUTx,

given studies in an allogeneic and xenogeneic fetal sheep model showing that a plateau of engraftment efficiency is eventually reached as one increases the dose of donor cells administered [84]. Along these same lines, another study also performed in the sheep model showed that administering busulfan to the pregnant ewe 6 days before IUTx led to a significant increase in donor cell engraftment [85]. Although instructive, it is doubtful that using a highly toxic myeloablative agent during pregnancy would be deemed acceptable in humans. Studies from MacKenzie's group demonstrated that it was possible to achieve therapeutic levels of engraftment after neonatal transplantation [86] with none of the cytotoxic effects of an agent such as busulfan, by selectively depleting the host HSC in utero using an antibody to the c-kit receptor. Although these two studies collectively support the notion that chimerism can be significantly enhanced after IUTx by emptying host HSC niches before donor cell infusion, additional studies are clearly needed to identify ways to optimize the beneficial effects on donor HSC engraftment while minimizing fetal and maternal risks.

Preclinical Animal Studies of In Utero Gene Therapy: Hemophilia A as a Model Genetic Disease for Correction by In Utero Gene Therapy

Multiple outstanding reviews have been written discussing IUGT in detail [5,6,87,88]. For this reason, in this section we will briefly highlight some of the key advantages and risks to this approach, focusing much of our discussion on hemophilia A (HA), using this disease as a paradigm to illustrate the utility of IUGT for treating or curing a wide array of genetic diseases. Gene transfer using viral vectors exploits the natural ability of the parent virus to attach to a target cell efficiently and transfer its genetic material to the host cell nucleus. However, viral vectors are engineered to be devoid of most if not all viral genes, thereby rendering the vector incapable of replication and avoiding the expression of potentially immunogenic and/or toxic viral genes. Although a complete vectorology discussion is beyond the scope of this chapter, the specific vector to be used for a given IUGT application will depend largely on one's goals and the disease and/or cell type being targeted and should be selected after careful consideration of factors such as the ability to integrate into host genomic DNA, tissue tropism, packaging capacity, and potential immunogenicity. An "ideal" vector should possess the ability to target a specific cell type/organ selectively and to mediate sufficient levels of gene transfer (and subsequent transgene expression) to produce a therapeutic effect with only a single treatment. Clearly, however, the ideal vector tropism will vary depending on the disease to be treated. As we will discuss in detail shortly, when targeting diseases such as the hemophilias, tissue-specific expression of the corrective gene is not required. As such, the use of a vector that is capable of widespread transduction and gene expression within the developing fetus is desired because it would lead to far greater therapeutic benefit.

When one considers initial target diseases for exploring the therapeutic potential of IUGT, the diseases that would be most amenable to treatment by IUGT are those caused by a mutation in a single gene. To contemplate in utero treatment, testing for the target disease must be in place to allow diagnosis before birth, and there must be compelling reasons to pursue prenatal treatment rather than waiting until after birth. Using a variety of animal model systems and rodent models of human genetic diseases, IUGT, using a wide variety of viral vectors, has been targeted to multiple organs. Moreover, in several disease models, phenotypic rescue has been accomplished with IUGT [87,89–97]. In the interest of brevity and for the purpose of illustrating the profound therapeutic potential of IUGT and the ease with which it could be implemented clinically to cure disease, the next section of this chapter will focus on HA, presenting HA as a paradigm for genetic diseases that could be corrected by IUGT, the rationale for pursuing its treatment before birth, the feasibility of doing so, and clinical, societal, and financial advantages IUGT could offer over existing treatments for this disease.

The Need for Better Hemophilia A Treatments

HA is the most common inheritable coagulation deficiency [98]. Its clinical severity varies based on factor VII (FVIII) plasma levels. However, up to 70% of patients with HA have a severe, life-threatening phenotype [99] and frequent spontaneous hemorrhaging, which leads to hematomas, chronic painful and debilitating arthropathies, and potentially life-threatening internal bleeding [99]. The current standard of care for HA is prophylactic factor infusion, which consists of intravenous infusions of recombinant or plasma-derived FVIII two to three times a week to maintain hemostasis. Although this "protein-replacement therapy" has greatly improved quality and length of life for many patients with HA, it is far from ideal. Patients are condemned to a lifetime of frequent intravenous infusions and are financially burdened with treatment costs that can exceed $300,000/year. Even among the approximately 25% of patients with HA worldwide who are fortunate enough to have access to FVIII prophylaxis,

approximately 30% will mount an immune response (inhibitors) to the infused FVIII [100]. In the best-case scenario, these inhibitors reduce the effectiveness of subsequent infusions of FVIII; in the worst-case scenario, they can lead to treatment failure, putting the patient at risk for a life-threatening bleed. These significant shortcomings highlight the need for novel therapies that can promise longer-lasting correction or permanent cure of HA.

In marked contrast to protein replacement, a single gene therapy treatment could promise lifelong correction of HA; indeed, several aspects of HA make it an ideal target disease for gene therapy [101,102]. First, FVIII does not need to be expressed in either a specific tissue or cell type to produce a therapeutic effect. Although the liver is thought to be the major site of FVIII synthesis within the body [103], FVIII can be expressed and perform its clotting function, as long as it is produced by cells that can release the synthesized FVIII into the circulation. Second, even if FVIII levels could be restored to only 3–5% of normal, this seemingly minimal change could exert a marked clinical effect in patients with severe HA, converting them to a moderate or mild phenotype and dramatically improving their quality of life [102]. This knowledge led the American Society of Gene and Cell Therapy to include the hemophilias in the most promising, "target 10" diseases in their roadmap (www.ASGCT.org).

Preclinical Animal Models to Study In Utero Gene Therapy for Hemophilia A

Fortunately, FVIII-deficient mice produced via gene targeting or knockout [104] and colonies of HA dogs in which spontaneous mutations occurred within the FVIII gene [105,106] are both available to study the biology of FVIII and to explore and develop gene-based strategies to treat HA. The phenotype in the HA mice is mild, in contrast to that of most human patients, and therapeutic benefit has been demonstrated in multiple postnatal murine gene therapy studies [107–115]. Postnatal gene therapy has also been shown to be capable of phenotypically improving HA dogs, but therapeutic benefit in this more clinically predictive model has proven far more difficult than in mice [116,117]. Despite these promising preclinical results, however, no therapeutic benefit has yet been seen in any of the clinical gene therapy trials that have been conducted for HA. This is in striking contrast to the successes in clinical gene therapy trials for hemophilia B (HB) [118]. Although the precise reasons for this marked difference in the ability of gene therapy to correct HA versus HB are not clear, this discrepancy is particularly vexing when one considers that roughly 80% of all clinical hemophilia cases are HA.

The difficulties seen thus far in translating success in animal models into therapeutic benefit in human patients highlight the importance of preclinical animal models that both precisely mimic the disease process of HA and closely parallel normal human immunology and physiology. To this end, we used a variety of reproductive technologies to reestablish and then clinically characterize a line of sheep successfully that possess a spontaneous frameshift mutation causing severe HA [119]. FVIII activity is undetectable in plasma of these sheep using a highly sensitive chromogenic assay, and if not treated at birth, these sheep die within the first hours or days of life [120,121]. All 10 affected animals that have been born have experienced multiple spontaneous episodes of severe bleeding, including muscle hematomas, hematuria, and hemarthroses, all of which have responded to infusion of human FVIII. In contrast to other animal models of HA, just like human patients with severe HA, these sheep experience frequent spontaneous bleeds into their "knees," which produce crippling arthropathies over time that ultimately lead to decreased movement, difficulties walking, and eventually symptoms of pain just to stand up. Also similar to human patients, some of these sheep developed inhibitors after administration of FVIII.

As discussed in detail in the section on IUTx, sheep possess many characteristics that make them an ideal preclinical model for IUGT. An additional unique advantage to using sheep in the context of HA treatment is that like humans, most of the FVIII carrier protein, von Willebrand factor (vWF), is stored or located within their platelets. This is in contrast to dogs, in which vWF circulates freely in plasma [122]. This important difference makes the sheep a highly relevant model in which to test the efficacy of platelet-targeted gene therapy approaches for HA [123].

Feasibility and Justification for Treating Hemophilia A Before Birth

Even if costs dropped sufficiently to enable most HA patients to afford FVIII prophylaxis, these patients would still require frequent, lifelong intravenous infusions and would still face the possibility of treatment failure owing to inhibitor formation. These problems, as well as many of the hurdles that have impeded gene therapy from curing patients with HA (and those with many other diseases as well), could be overcome or eliminated by performing gene therapy in utero. In 75% of HA cases, a family history exists that makes it possible to screen before birth. Prenatal diagnosis for HA is feasible and available, and is both encouraged and cost-effective, even within developing countries [124,125]. Moreover, it is now possible to diagnose HA in utero by performing digital polymerase chain reaction (PCR) on cell-free fetal DNA present in the mother's blood, which makes the prenatal diagnosis of HA effectively risk-free [126]. Despite the availability of prenatal screening, however, about 1 in 5000 boys born throughout the world each year have HA [98]. Corrective IUGT could remove the heavy physical, psychological,

and monetary burden on patients, their families, and the health care system. The estimate for the lifetime cost of prophylactic treatment for one HA patient is $20 million. Curative IUGT would thus save approximately $4.8 billion over the lifetime of the patients with HA in the United States.

Whereas after birth any intervention places a patient with HA at risk for bleeding, during early fetal life, activation of factor X occurs primarily via tissue factor activity, making it largely independent of the factor IXa (FIX)–FVIIIa phospholipid complex [127]. As a result, the fetus develops without hemorrhage despite having little or no expression of FVIII and FIX [127–129]. The unique hemostasis of the fetus should thus allow IUGT to be performed safely for HA; indeed, 1 of the 46 human patients who received IUTx was transplanted in the hope of correcting HA, or at least inducing immunological tolerance to FVIII [130]. Although only this one patient with HA was treated, he experienced no untoward effects as a result of the in utero intervention, he exhibited a reduced severity of disease compared with his siblings, and in contrast to his siblings, he did not develop inhibitors with FVIII treatment [130]. This remarkable case thus provides clinical validation for prior experimental studies demonstrating that exposure to vector-encoded proteins (including coagulation factors) during early immunologic development induces stable immune tolerance [131–134]. The lifelong tolerance to FVIII induced by an IUGT-based HA treatment should therefore prevent the development of FVIII inhibitors that plague patients treated with replacement therapy [121,135]. In this one clinical case, Dr. Touraine relied on the ability of unpurified fetal liver cells to produce sufficient levels of FVIII endogenously, after transplant, to mediate correction. The only partial correction that was observed in this patient supports the approach of using gene transfer to ensure adequate levels of FVIII are obtained for full phenotypic correction.

We showed that by administering a single intraperitoneal injection of a small volume of γ-retroviral vector during gestation, it is possible to achieve gene transfer levels within the hematopoietic system of 5–6% [94,136,137], levels that would certainly be beneficial in HA. Subsequent studies in which we selected $CD34^+$ cells from these IUGT recipients and performed serial transplantation or repopulation [94,137,138] provided compelling evidence that this approach successfully modified bona fide HSC, indicating this method could provide lifelong disease correction.

Results from these studies also demonstrated that hepatocytes and hepatic endothelium were transduced at levels that would be therapeutic in HA and defined the period of gestation for optimal transduction of these cells [93]. IUGT experiments performed by other investigators during the same period using sheep, rodent, and nonhuman primate models, and employing a variety of viral vectors, produced similar results [87,89,90,93,94,132,133,139–154]. These collective results clearly support the ability of this method to deliver an FVIII transgene to the nascent liver with adequate efficiency to convert patients with severe HA to a moderate or perhaps even mild phenotype.

As discussed at the beginning of this section, one of the most frequent and most dangerous clinical problems when treating severe HA by protein replacement is the formation of inhibitors in about 30% of patients. We have spent several decades performing IUGT studies in the sheep model and have demonstrated that it is possible to take advantage of the unique temporal window of relative immunonaivety during early gestation to deliver exogenous genes efficiently to a variety of fetal tissues and induce durable cellular and humoral tolerance to vector-encoded gene products [131]. Further mechanistic studies demonstrated that early in fetal development, IUGT exploits both central and peripheral tolerogenic avenues in the fetus [134]. These results strongly imply that even if it is not curative, IUGT would still be an ideal approach to treating HA, because the state of immune tolerance induced by IUGT would ensure that postnatal therapy, whether it is protein- or gene-based, could proceed safely with none of the immune-related problems that plague HA treatment.

Interestingly, although the incidence of HA is about seven times that of HB, the only experimental studies to test the ability of IUGT directly to correct the hemophilias have targeted HB (factor IX [FIX] deficiency) [89,132,133,139,150,152–156]. The choice to target HB rather than A most likely results from the greater ease with which FIX can be cloned into a variety of viral vectors and efficiently expressed upon transduction of appropriate target cells. The treatment of HB by IUGT has been extensively studied in murine models, with different groups performing gene transfer at differing gestational ages via different routes of injection and using different vector types. Sabatino et al. reported low-level human FIX expression after intramuscular injection of fetal mice with either AAV-1 or AAV-2. As predicted, the injection of AAV-1 induced tolerance and allowed the postnatal readministration of the FIX-encoding AAV-1 vector, increasing FIX levels sufficiently to reach the therapeutic range. Surprisingly, however, injection of AAV-2 did not induce immune tolerance [157]. In other studies, Schneider et al. compared various routes of administration for delivering adenovectors and AAV-2 vectors encoding human FIX into mouse fetuses and found that adenovectors resulted in initially higher levels of FIX. Mice injected in utero with nonintegrating adenovectors also maintained therapeutic levels of FIX for 6 months, and no antibodies developed against either vector or transgene [154].

Without a doubt, Waddington and colleagues achieved the most clinically promising results of IUGT in hemophiliac mice by showing that the injection of an FIX-encoding lentiviral vector into embryonic day 15 mouse fetuses resulted in levels of FIX that were 9–16% of normal and improved coagulation for 14 months after IUGT. Furthermore, no immune response developed to FIX, even when the authors attempted to break tolerance by repeatedly injecting the protein postnatally [133].

Collectively, these murine studies have provided compelling evidence that IUGT can result in the expression of FIX at levels that not only have therapeutic significance but are often sufficient to induce tolerance, thus allowing postnatal administration of the same vector or the FIX protein without eliciting an immune response. Because patients with HA have at least a 10-fold higher likelihood of developing inhibitors than do HB patients [158,159], these studies, although encouraging, leave unanswered the critical question of whether IUGT will also induce stable immune tolerance to FVIII, given its higher inherent immunogenicity.

Risks of In Utero Gene Therapy

Despite the great promise IUGT holds for treating the many genetic diseases that can be diagnosed prenatally, several important safety concerns must be addressed before its clinical application. There are two sets of potential safety concerns when contemplating IUGT: those associated with fetal intervention and those owing to the gene transfer itself. As with any fetal intervention, infection, preterm labor, and fetal loss are all theoretically possible. In reality, however, a wealth of clinical data exist demonstrating that the human fetus can be safely accessed multiple times with an extremely low procedure-related risk [75,88]. Any manipulation of the fetus obviously has the potential to alter normal organ development, and the possibility for deleterious effects owing to the injection and from any inherent toxicity of the vector itself need to be considered and carefully evaluated. Nonhuman primates injected with lentiviral vectors in utero via either the intrapulmonary or intracardiac route showed no adverse effects on postnatal heart and lung development [148], which suggests that IUGT exerts minimal if any effects on organ development. In contrast, Flake and colleagues reported that expression of fibroblast growth factor-10 in the developing rat lung after IUGT led to cystic adenomatoid malformations; this illustrated how forced expression of a specific transgene can lead to malformation [160]. These findings suggest that strategies involving the expression of growth factors, transcription factors, or other regulatory molecules will need to be carefully examined because they may have significant potential to alter normal organ development, particularly early in gestation. One study also showed that AAV vectors administered to the fetus can cross the placenta, enter the maternal circulation, and transduce multiple tissues within the mother [156]. Because this study was performed in nonhuman primates, whose placentation is similar to that of humans, this is an issue that will likely need to be explored in greater detail and with other commonly employed vectors to better define or quantitate the risk of inadvertent gene transfer to maternal tissues and ascertain what risk, if any, this will pose to the mother.

In addition to the general risks associated with fetal intervention, two unique risks have raised the most concern regarding the use of IUGT: vector-mediated insertional mutagenesis and germline transmission [161]; each of these will now be discussed.

Genomic Integration-Associated Insertional Mutagenesis

Insertional mutagenesis is a major concern with all of the integrating viral vectors and has been subjected to intense scrutiny since four cases of T-cell leukemia occurred in human pediatric patients with X-SCID 31–68 months after they received γ-retroviral vector-modified autologous HSC. This concern was further heightened when linker-mediated PCR analysis of lymphocytes from these patients revealed that insertional mutagenesis had occurred in all four patients and was at least partially responsible for the observed leukemogenesis [162–164]. A later study in which genotoxicity or leukemogenesis was also observed after γ-retroviral or HSC-based gene therapy to treat Wiskott–Aldrich syndrome [165] heightened this concern. Importantly, in our long-term IUGT studies in fetal sheep, we also employed γ–retroviral vectors and achieved significant levels of gene transfer to bona fide HSC [94,136–138], yet we did not observe leukemogenesis in any of these animals. Because sheep have a life span of roughly 10 years, this study should more or less approximate a 35-year follow-up in "human years." A major difference between our study and the clinical trial that likely explains the markedly different outcome is the transgene that was delivered via the retroviral vector. In our experimental proof-of-concept studies, we employed marker genes to enable us to track and quantitate transduction and transgene expression easily in various tissues. In the clinical trial for X-SCID, the vector encoded the therapeutic common γ chain (γc) gene (interleukin-2 receptor subunit γ), because this was the gene defect causing X-SCID. Subsequent studies revealed that the observed leukemogenic event in these patients likely arose from a combinatorial effect of both the insertion of the vector in close proximity to the LIM only protein 2 oncogene and a growth advantage that was conferred on the transduced

cells expressing high levels of the therapeutic γc gene [166–169]. This clinical trial thus provides a valuable lesson in the complexities of risk assessment in gene therapy, which is still a relatively new and rapidly evolving field.

Looking specifically at IUGT, there has been only one report of oncogenesis after in utero delivery of a viral vector. In these studies, Themis, Waddington, Buckley, and colleagues reported a high incidence of postnatal liver tumors in mice after prenatal injection with a third-generation equine infectious anemia virus–based lentiviral vector. These tumors were not seen in mice that received a nearly identical vector that was constructed on an HIV backbone [170–172]. The authors did not identify the genomic insertion sites in these animals, so it remains unclear whether insertional mutagenesis was the cause of the observed tumor formation. Nevertheless, this important study demonstrates that the fetus may be particularly sensitive to tumorigenesis induced by certain vectors. The findings of Themis and the results of the clinical trial for X-SCID collectively suggest that preclinical assessment of the risk of insertional mutagenesis after IUGT will require carefully designed preclinical studies with the actual vector to be employed for the pending clinical trial in an animal model that has been thoroughly validated in the setting of the target disease.

Potential Risk to Fetal Germline

Although gene transfer to the vast majority of fetal tissues would be desirable for correcting diseases that would benefit from widespread systemic release of a secreted transgene product (such as HA), gene transfer to cells of the developing germline would raise major ethical issues. In the human fetus, the primordial germ cells (PGC) are compartmentalized within the gonads at 7 weeks of gestation [173]. The germline should be accessible only through the vascular system, so targeted gene therapy that is administered after this period in gestation should not affect the germline. Nevertheless, the possibility of inadvertent gene transfer to the germline is clearly a major safety concern and a bioethical issue, and studies have demonstrated that both the embryonic germline [174–177] and isolated PGC [178] can readily be infected with γ-retroviral vectors and pass the vector genetic material to subsequent generations in a Mendelian fashion as part of the permanent genome. For this reason, we used a three-tiered approach to examine this important issue in detail in the sheep model of IUGT. We performed (1) immunohistochemical staining on reproductive tissue from the in utero–treated animals, (2) genetic analysis on the sperm cells from the treated males, and (3) breeding experiments in a limited number of animals [94,137,141,142]. These studies indicated that although the fetal ovaries appeared to be largely unaffected by this approach to IUGT, numerous cells within the developing fetal testes were modified, including interstitial cells, Sertoli cells, and small numbers of both immature germ cells within the forming sex cords and the resultant sperm cells. Importantly, however, gene-modified germ cells were observed in only two of the six animals examined, and the incidence of germ cell modification in these two animals was about 1 in 6250, a frequency that is well below the theoretical level of spontaneous mutation within the human genome [179]. This low frequency of modification, coupled with observations that genetic alterations to the germ cells may produce deleterious effects, placing them at a disadvantage during fertilization, suggests that the likelihood that any genetic alterations present would be passed to subsequent offspring would be extremely unlikely. In agreement with this supposition, we did not observe transfer of the vector sequences in any of the 10 offspring we studied, even when mating animals that had both received IUGT. Nevertheless, this is clearly an issue that will need to be addressed in greater detail before clinical application of IUGT. This need for further investigation was underscored in other IUGT studies performed by Tarantal and colleagues, who employed lentiviral vectors in nonhuman primates and observed modification of the female germline but no effect on the male germ cells [140]. Thus, the issue of germline safety will likely have to be investigated in more than one preclinical model, employing the specific vector being considered for clinical use, to obtain an accurate assessment of the risk posed by the procedure.

Genome Editing

The field of gene therapy is rapidly advancing, and the development of genome-editing technologies such as zinc finger nucleases, transcription activator-like effector nucleases, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated genes [180,181] promises to revolutionize the way in which gene therapy is conceptualized. The ability to modify a specific sequence of DNA in its native genomic locus offers remarkable advantages in terms of both safety and efficacy, over current "gene addition" and should largely eliminate existing concerns related to random genomic integration, inappropriate levels or tissue distribution of transgene expression, and inadvertent germline alteration. One would therefore expect that these newer gene-editing technologies will be an important component of future IUGT studies and trials. However, these systems will likely introduce their own unique set of risks and concerns, and further study will be required to define and fully understand what these risks may be and evaluate whether the benefits these systems can offer over "traditional"

viral vectors outweigh these risks. In addition, well-designed experimental studies in suitable preclinical models will be required to determine whether these genome-editing systems should be administered directly to the recipient to mediate gene correction (which will likely require the use of viral vectors to achieve sufficient efficiency), or whether modifying suitable cell populations in vitro, followed by the infusion of these gene-corrected cells, will prove the safer mean to move these technologies toward clinical application.

CLINICAL EXPERIENCE WITH IN UTERO STEM CELL TRANSPLANTATION

Early successes with experimental IUTx in animals discussed in the preceding sections quickly led to attempts around the world to treat various human hematologic disorders with IUTx. In 1989, Touraine performed the first successful human IUTx for bare lymphocyte syndrome [182]. After this seminal case, several centers reported the successful transplantation of fetuses with SCID [183–186] using a variety of fetal and adult sources of HSC and transplanting the fetus between 16 and 26 weeks' gestation. In each case, IUTx resulted in readily detectable engraftment of donor cells at birth and clinical improvement. IUTx has been performed on 46 human patients for 14 different genetic disorders [4], collectively providing definitive proof that the early human fetus can be accessed multiple times with an extremely low procedure-related risk [75,88].

Although the successful treatment of immunodeficiencies provided clinical proof of the vast therapeutic potential of IUTx, the clinical experience thus far with IUTx for a wide range of other disorders has unfortunately been largely disappointing. The tremendous success with SCID is a function of the unique nature of this disorder, which provides a survival and proliferative advantage for donor HSC-derived T cells. Indeed, in these patients, the only hematopoietic lineage reconstituted by the donor cells has been the T-cell lineage (split chimerism) [88], echoing the findings in Blazar's early murine studies [32]. The results of the 46 clinical IUTx cases have proven that using current methods, IUTx is unable to achieve therapeutic levels of engraftment in recipients whose hematopoietic system possesses a normal level of "competitiveness." That these transplants were performed at different centers, at variable gestational ages, and used donor cells from different sources and/or with different phenotypes, has made it difficult to identify common factors that may be responsible for the poor levels of donor engraftment. The inherent inconsistency in these studies has made it necessary to perform many of the more carefully controlled animal studies that were previously described to identify the nature of the barriers that limit engraftment after IUTx and to develop means of overcoming them to achieve clinical success.

The hematopoietic system and marrow microenvironment of patients with hemoglobinopathies, lysosomal storage diseases, and the vast majority of the other disorders that are being considered for treatment with IUTx are competitively normal during fetal development. As such, clinical success with IUTx for these diseases will require the development of methods to overcome endogenous host cell competition. To this end, Flake and colleagues showed [53] in the canine model that administering large doses of highly enriched HSC via a novel ultrasound-guided, intravascular (intracardiac) route produced significantly higher engraftment levels than the intraperitoneal route that has traditionally been employed for IUTx in human patients. This study generated a good deal of excitement in the field, because the levels of donor cell engraftment the authors obtained with the new route would likely be therapeutic in most candidate diseases. However, studies performed by Tanaka et al. in the sheep model have shown that the intravascular route is no better than the intraperitoneal route, which led the authors of this other study to conclude that the markedly greater safety afforded by the intraperitoneal route will likely make this the clinical route of choice [187].

One particular area that has received a great deal of attention is the idea that the best immediate clinical application for IUTx may be to use it to induce prenatal tolerance, thus making it possible to perform a curative postnatal BM transplantation without the need for highly toxic cytoablation and immunosuppression [6,13]. This approach greatly lowers the threshold of donor cell engraftment that would be required for clinical success, because, as discussed previously, induction of donor-specific immune tolerance can reliably be obtained with stable levels of donor chimerism of only 1–2%. The therapeutic merit of this approach was experimentally validated by Peranteau et al [26]. In this landmark study, the authors demonstrated that low-level hematopoietic engraftment to induce tolerance, followed by postnatal nonmyeloablative same-donor "boosting" BM transplantation, resulted in high levels of donor cell engraftment and phenotypic correction in murine models of β–thalassemia and sickle cell disease, two diseases that had previously proven completely refractory to correction by IUTx. These promising results led the authors to conclude optimistically that "if adequate engraftment can be achieved to consistently induce donor-specific tolerance without [graft-versus-host disease] in a preclinical model, then clinical trials of

IUTx for treating genetic disorders that can be prenatally diagnosed and treated by mixed hematopoietic chimerism, such as the hemoglobinopathies and selected immunodeficiency disorders should be initiated" [26].

CONCLUSIONS AND FUTURE DIRECTIONS

Great progress has been made over the past few decades in the fields of IUTx and IUGT. However, multiple hurdles still need to be overcome for these therapies to become well-established clinical approaches. Challenges for IUTx are primarily related to overcoming the competitive barriers to engraftment in the "hematopoietically fit" fetus and better defining the innate and adaptive immune limitations to engraftment in large animals and humans. As our understanding of stem cell biology, developmental hematopoiesis, and the ontogeny of the hematopoietic niche advance, the therapeutic applications of IUTx will likely expand from their narrow focus to include the treatment of nonhematopoietic diseases. Although the strategy of prenatal tolerance induction for the facilitation of postnatal HSC transplantation is nearing clinical application and has great potential to benefit many patients, the development of an IUTx strategy that allows a single-step treatment to achieve therapeutic levels of engraftment would be ideal and would likely propel this promising therapy into the clinic.

IUGT holds even greater promise for treating and curing essentially any inherited genetic disease. From the results of countless studies exploring IUGT in sheep, mice, and nonhuman primates, it is clear that the direct injection of viral vectors into the developing fetus can efficiently deliver an exogenous gene and achieve long-term expression in multiple tissues. As such, one can envision a day when IUGT becomes a viable therapeutic option for diseases affecting any of the major organ systems. Importantly, even if it is not curative, IUGT would be an ideal approach for diseases such as HA, because lifelong immunologic tolerance could be induced to the missing FVIII protein, thus overcoming the immune-related hurdles that hinder postnatal treatment of this disease. Despite its great potential, however, IUGT is still in the experimental stages, and several important safety concerns need to be extensively investigated in appropriate preclinical animal models before initiating studies in human patients.

IUTx and IUGT stand at a critical juncture [188] and have vast potential for dramatically improving human health care. Many of the most intimidating obstacles have been overcome in animal models or are at least better understood, which has revitalized this exciting field. There is no doubt that surpassing the few remaining hurdles to allow clinical implementation of these therapies will dramatically change the whole paradigm for the way in which the world perceives and treats genetic disorders.

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CHAPTER

58

Engineering of Large Diameter Vessels

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INTRODUCTION

Cardiovascular diseases (CVDs) such as aortic disease, coronary artery disease (CAD), and peripheral artery diseases (PAD) are the leading causes of mortality worldwide [1]. In 2011, the age-standardized death rate attributable to all CVDs was 229.6 per 100,000 in the United States. Based on 2011 death rate data, more than 2150 Americans die of CVDs each day, an average of one death every 40 s [1]. Atherosclerosis is one of the most important causes of CVDs; it induces stenosis, occlusion, and calcification of arteries in patients with CAD and PAD. Aortic aneurysm or dissection often occurs in patients with aortic disease. In addition, chronic kidney disease is reaching epidemic proportions because patients have risk factors for CVDs such as hypertension, hyperlipidemia, smoking, and diabetes. Many patients with end-stage renal disease (ESRD) require vascular shunts for hemodialysis. Clearly, CVDs impair the quality of life for many individuals.

There are various therapeutic strategies for CVDs. In particular, lifestyle modifications and pharmacological therapies have important and fundamental roles in treating CVDs. In addition, more invasive endovascular intervention such as thrombectomy, balloon dilatation, stent implantation, and/or surgical procedures may be recommended for more severe cases of CVDs. Surgical procedures typically involves graft replacement and/or bypass procedure. In CADs, patients with left main coronary disease or three-vessel disease typically undergo percutaneous coronary intervention or coronary artery bypass grafting (CABG). Second-generation drug-eluting stents have been developed to improve patency and reduce major adverse cardiac events, but CABG should remain the standard therapy for high-risk patients [2]. Common graft sources for CABG are the autologous bilateral internal thoracic arteries or the saphenous vein. In contrast, the use of a synthetic vascular graft such as expanded polytetrafluoroethylene (ePTFE) (Gore-Tex) or polyethylene terephthalate (Dacron) is common for patients with aortic disease. In PAD, autologous venous grafts are primarily used for peripheral arteries below the knee, whereas synthetic grafts also are used for larger arteries such as iliac, femoral, and popliteal arteries (above the knee). In ESRDs, typical vascular access for hemodialysis is provided by autologous arteriovenous (AV) shunts. Graft selections depend on the patency. In smaller arteries such as the coronary artery and below-the-knee arteries, synthetic grafts have shown unfavorable results owing to high incidences of thrombosis. In medium-size arteries, such as the femoral and popliteal arteries, polytetrafluoroethylene (PTFE) grafts have shown patency rated of 60% and 27% compared with 89% and 85% when using the saphenous vein at 1 and 5 years, respectively [3]. Therefore, it is recommended that synthetic grafts be used only when autologous grafts are unavailable. In aortic diseases, there are no suitable autologous substitutes. In general, synthetic materials have shown limitations associated with thrombosis, stenosis, calcification, infection, a lack of durability, the need for anticoagulation therapy, and the inability to regenerate native tissue. Suitable autologous tissues are in short supply, which makes it difficult to perform multiple or repeat operations.

To overcome the limitations associated with autologous and synthetic grafts, the concept of the tissue engineered vascular graft (TEVG) was proposed. Vacanti et al. defined tissue engineering as the fabrication of alternative materials that integrate with a patient's native tissue for the purpose of restoring biological and physiologic function [4]. In 1986, the first tissue engineered blood vessel was reported by Weinberg and Bell and consisted of bovine endothelial cells (ECs), smooth muscle cells (SMCs), and fibroblast cultures embedded in a collagen gel integrated with a Dacron mesh [5]. To date, hundreds of TEVGs have been developed and evaluated in animal models and

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clinical studies. It is promising technology for pediatric cardiologists who treat congenital heart diseases because TEVGs can regenerate and remodel new vessels and provide growth potential. In 2001, the first TEVG clinical trial was performed in children with congenital heart disease [6]. Similarly, TEVGs have attracted attention from nephrologists looking for alternatives to AV shunts. Niklason et al. reported that their clinical study, which used TEVGs to provide hemodialysis access, produced favorable safety and patency results [7]. In this chapter, we look at the status of large-diameter TEVGs in large-animal models and clinical studies and share the outlook for the future of TEVGs.

MATERIALS FOR AND APPROACHES TO FABRICATING TISSUE ENGINEERED VASCULAR GRAFTS

A large number of fabrication materials have been proposed to construct large-diameter TEVGs that have the potential to integrate with and remodel into native. There are currently three strategies regarding materials, scaffold, and TEVGs: (1) biological-based, (2) biodegradable synthetic-based, and (3) hybrids (Fig. 58.1). A large number of biological-based and biodegradable synthetic-based matrix combinations have been evaluated, but few have been applied clinically.

Biological-Based Scaffolds

When designing and constructing an arterial TEVG, it must be durable enough to withstand high arterial pressures. With this in mind, the use of natural matrices from animal or human arteries may provide advantages with regard to structure and mechanical properties. However, when xenogenic and allogenic matrices are transplanted, immunological rejection often sets in. Therefore, they require a decellularization process to avoid adverse immunological reactions.

Natural Decellularized Matrices

Xenogenic Matrices

Vessels and nonvascular tissues harvested from animals or cadavers are two distinct sources when fabricating decellularized vascular matrices. Decellularized vascular grafts sourced from animals or cadavers have a structure similar to native vessels; therefore, they have inherent advantages with regard to mechanical properties. Nonvascular tissues such as the small intestinal submucosa (SIS) and amniotic membrane also contain natural extracellular matrix (ECM). However, the SIS and amniotic membrane must be shaped into tubular constructs. They have been used as the materials for TEVGs, but neither have reached clinical trials or preclinical animal studies to investigate their efficacy as large-diameter TEVGs. All nonautologous natural matrices require decellularization to remove most cellular antigenic components. Different decellularization methods have been proposed and typically involve a combination of physical approaches and chemical and biological agents. Physical approaches include freeze-thawing, mechanical abrasion, hydrostatic pressure, and nonthermal irreversible electroporation [8]. Acids and bases, hypotonic and hypertonic solutions, detergents, and alcohols are common chemical agents. Enzymes such as DNases and trypsin, nonenzymatic agents such as ethylenediaminetetraacetic acid, and serine protease inhibitors such as phenylmethylsulfonyl fluoride are common biological agents. The ideal combination of decellularization methods and agents must be selected carefully and will successfully maintain the three-dimensional (3D) structure and mechanical properties of ECM while removing cell remnants. Therefore, the merits and limits of each respective method must be understood.

Decellularized vessel grafts have the longest history with regard to the development of TEVGs. Since the 1960s, many decellularized TEVGs have been developed and some have been marketed commercially. Most of these TEVGs have been produced for AV shunts. The most popular decellularized vessel graft that is available commercially is Artegraft. This xenogenic TEVG is composed of a decellularized bovine carotid artery graft for hemodialysis access; the primary patency rate was significantly higher than in ePTFE grafts at 1 year (60.5% versus 10.1%; $P = .0062$) [9]. Furthermore, fewer percutaneous angioplasties were performed in upper-arm Artegrafts than in ePTFE grafts (1.30 ± 0.27 versus 2.49 ± 0.43 times per patient-year; $P = .014$). Therefore, the authors concluded that Artegrafts were an excellent option for hemodialysis AV shunts. Another popular xenogenic graft is SynerGraft Model 100, which is based on decellularized bovine ureters [10]. In a clinical study, 29 patients were implanted with SynerGraft and 27 received ePTFE grafts as AV shunts; they showed similar patency (28% versus 48%; $P = .290$) and infection rates (4% versus 9%; $P = .410$) between SynerGraft Model 100 and ePTFE after

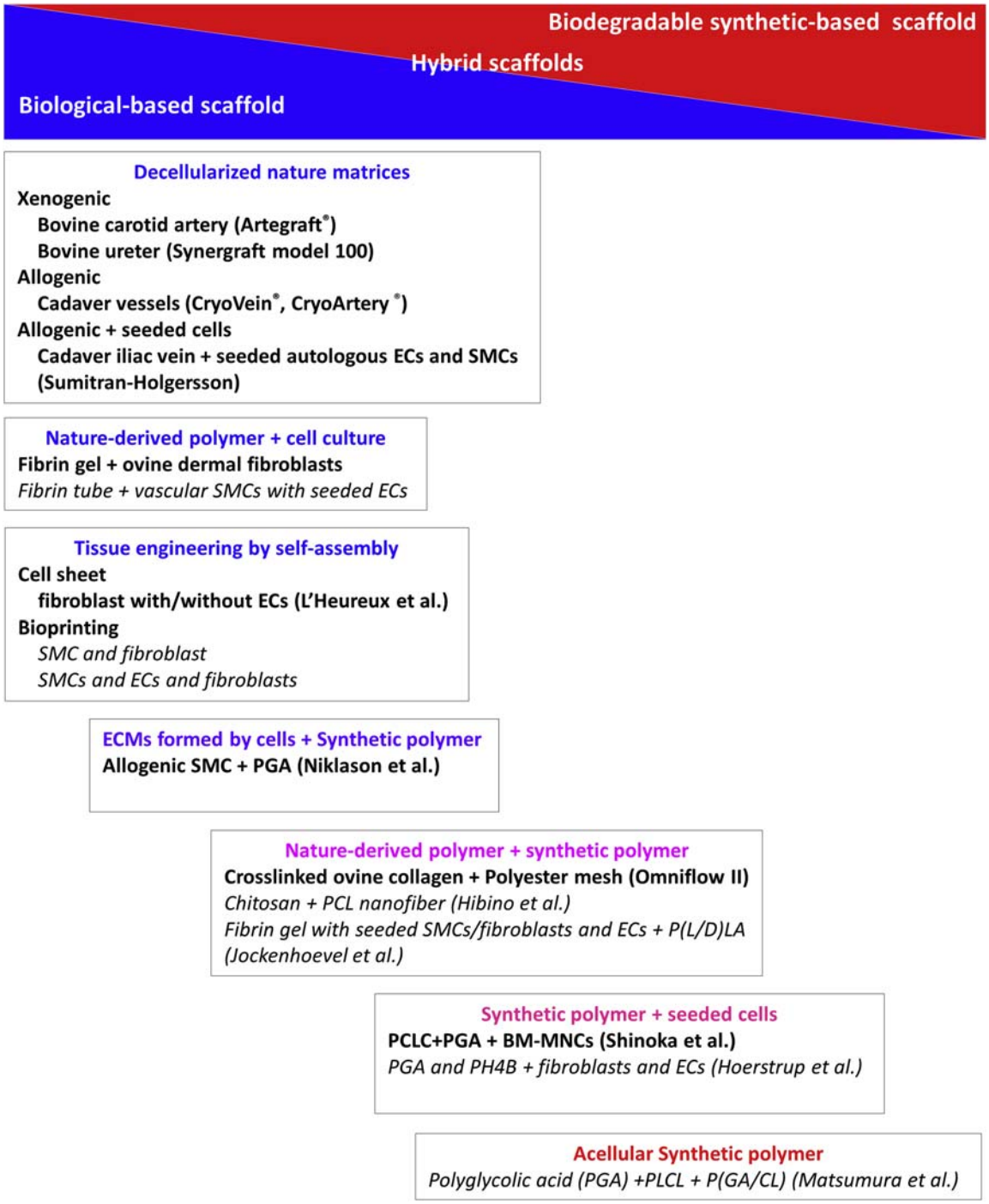

FIGURE 58.1 Large-diameter graft classification by scaffold materials in large-animal model and clinical studies. Various combinations of biological-based scaffolds and biodegradable synthetic-based scaffolds have been developed and evaluated in large-animal models and clinical studies. Large-animal model studies are shown in *italics* and clinical studies are shown in *bold*. *BM-MNCs*, bone marrow mononuclear cells; *EC*, endothelial cell; *PH4B*, poly-4-hydroxybutyrate; *PCL*, poly(ε-caprolactone); *PGA*, polyglycolic acid; *P(GA/CL)*, poly(glycolide-*co*-ε-caprolactone); *PLCL*, poly(L-lactide and ε-caprolactone); *P(L/D)LA*, poly(L/D)lactide; *SMC*, smooth muscle cells.

1 year. Unfortunately, advantages for SynerGraft Model 100 were not seen. To improve patency, decellularized xenogenic grafts seeded with cells have been developed in animal models. For example, in ovine models, decellularized porcine carotid artery grafts seeded with endothelial progenitor cells (n = 6) remained patent up to 4.4 months as AV shunts [11].

SIS may be a promising source of TEVG matrices. SIS is a kind of natural ECM that has been shown to retain a porous scaffold suitable for tissue reconstruction. The porous scaffold allows for easy cell infiltration. SISs have been applied in many clinical areas such as the ileum, diaphragm, rotator cuff, and bladder [12–14]. Various SIS-derived TEVGs have been developed. In a large-animal study, 24 SIS grafts obtained from pigs were implanted as ovine carotid arteries [15]. However, patency at 3–4 months was 30%. In general, the patency rates of decellularized xenogenic grafts do not exceed those of ePTFE in many clinical and animal studies. Andreadis and Swartz et al. reported that SIS TEVGs coated sequentially with heparin and vascular endothelial growth factor showed successful endothelialization, remodeling, and development of vascular function in ovine carotid artery models [16]. The patency was 92% in 12 sheep at 1 weeks (n = 3), 1 month (n = 4), and 3 months (n = 4). However, SIS grafts have not been used clinically.

Allogenic Matrices

Allogenic vascular grafts are provided by human cadavers. CryoVein, which is composed of cryopreserved saphenous vein allografts, has been evaluated for many years. Farber et al. reported the results of 240 infrainguinal revascularization procedures with CryoVein [17]. The primary patency rate was not sufficient (50% at 6 months and 18% at 24 months) [17]. Similarly, CryoGraft has been used for hemodialysis access, compared with PTFE shunts, and revealed a similar primary patency at 2 years between the two groups. CryoGraft showed more resistance to infection but was also more susceptible to aneurysm [18]. Cryopreserved arteries (CryoArtery) have been developed to treat infected aortic prosthetic grafts or mycotic aneurysms. Evaluations of CryoArtery revealed an overall treatment mortality rate of 21% at 12.5 months, and graft-related complications were acceptable at midterm follow-up regardless of the disease severity risk [19]. An advantage to using decellularized allogenic and/or xenogenic matrices is that they can address the limited availability of autologous grafts, but the advantage is limited by their lack of cellularity. The lack of cellularity may lead to graft thrombosis, infection, and aneurysm. Therefore, cell seeding technologies have been developed to modify grafts. In a first case report, a cadaveric allogenic iliac vein graft seeded with autologous bone marrow–derived ECs and SMCs was implanted into a patient with extrahepatic portal vein obstruction as a bypass between the superior mesenteric vein and the intrahepatic left portal vein (meso Rex bypass) [20]. The graft was patent at 9 months after the operation but narrowed owing to mechanical obstruction by the surrounding tissue and required a second operation to be performed. The authors stated that the advantage of this graft is that it is unnecessary to use immunosuppression therapy because the decellularized allogenic graft is seeded with autologous cells. However, human donor cadaver vessels are in short supply and result in complicated ethical issues. In addition, inadequate decellularization has caused cases of adverse immune response and graft failure. On the other hand, aggressive decellularization may destroy the structural integrity of the ECM and lead to decreased mechanical properties. Therefore, allogenic vessel-derived TEVGs are not commercially widespread.

Nature-Derived Polymers

ECMs are composed of collagen, proteoglycan, fibronectin, elastin, etc., and therefore are good candidates as TEVG scaffold materials. Many researchers have studied these different nature-derived polymers as TEVG scaffolds. In sheep models, Tranquillo et al. fabricated 4-mm-diameter grafts composed of ovine dermal fibroblasts and fibrin gel. The fibrin gel from bovine fibrinogen and ovine dermal fibroblasts was combined and cultured into a tubular mold for 2 weeks and the grafts were subsequently transferred to a pulsed flow-stretch bioreactor for an additional 3 weeks and then decellularized. The grafts were implanted interpositionally in the femoral artery of six sheep; all were patent and showed no dilatation or calcification at 24 weeks [21]. In addition, the researchers implanted grafts fabricated in the same manner to replace the pulmonary artery in three young lambs. The lambs displayed normal growth after 50 weeks [22]. Andreadis et al. also reported a conceptually similar TEVG in which fibrin tubes with entrapped vascular SMCs were implanted as vein interposition grafts in lambs [23]. The graft was seeded with ECs in the luminal TEVG surface before implantation; subsequently, they showed patency for 15 weeks. Silk-derived fibroin, collagen, and chitosan are also potential nature-derived polymers as TEVG scaffolds. However, these combination strategies have been evaluated only in vitro and in small-animal model.

Tissue Engineering by Self-assembly

The Tissue Engineering by Self-Assembly (TESA) design approach, which L'Heureux et al. developed, is a promising and unique fabrication method of TEVGs [24]. TESA does not use nature-derived polymers or synthetic scaffolds but is based on cell sheet tissue engineering. The cell sheets are formed by culturing autologous fibroblasts from a biopsy. Cell sheets are wrapped around a 4.8-mm-diameter stainless-steel mandrel and then matured. After taken off the mandrel, the TEVG is dehydrated and luminally seeded with autologous ECs. The production time for

these grafts ranges from 6 to 9 months. According to published data, the first 10 patients underwent AV shunt graft implantation and primary patency rates were 78% at 1 months (7/9) and 60% at 6 months (five of eight patients; one patient withdrew and another patient died of unrelated causes) [25]. The authors stated that this patency was acceptable because patients enrolled in the study had a high probability of failure and had at least one previous access failure. Thus, the study group represented a particularly challenging patient population in which AV shunt failure was expected to be far higher. L'Heureux et al. also reported that nonendothelialized TEVGs were implanted into three patients as AV shunts [26]. Two patients required intervention for stenosis and occlusion (both eventually failed) within a year and one patient died of sepsis. The authors suggested that an endothelium may not be required to maintain the patency of this TEVG because thrombogenic failures appeared to result from stenosis. Removing the EC seeding process simplifies the fabrication of TEVGs and saves on cost and time. Furthermore, they developed a novel assembly method termed thread-based tissue engineering (TBTE) to save production time compared with TESA. TBTE technology uses the cell-synthesized matrix of fibroblasts to form strong threads that can be assembled into complex 3D structures using classic textile technologies such as weaving, braiding, or knitting. Preclinical studies are investigating TBTE grafts for canine AV shunt and CABG [27].

Other TESA technologies include microtissue aggregation and 3D cell printing. Forgacs et al. fabricated prototype biological self-assembly bioprinted grafts in vitro [28]. Multicellular spheroids or cylinders 300−500 μm in diameter were made from SMCs and fibroblasts. Subsequently, agarose rods and the uniform multicellular spheroids were deposited layer by layer. With this method, various tubular and branching structures can be fabricated into TEVGs. Itoh et al. manufactured another type of 3D bioprinting TEVG using multicellular spheroids and implanted the constructs into rat abdominal aorta ($n = 5$) [29]. Their unique method skewered the multicellular spheroids into a needle array according to a predesigned 3D structure. They implanted the grafts into rat aortas and evaluated their histologies 5 days after implantation. These technologies have not yet reached clinical application. The major limitations of these technologies are that they require long times to culture and they have difficulty attaining suitable mechanical strengths. However, the technologies can fabricate bifurcated TEVGs or grafts with different-sized ends. Therefore, many researchers are continuing to investigate them.

Hybrid Scaffolds

Hybrid scaffolds combine biologically based and biodegradable synthetic materials. Many synthetic materials have been used as TEVG scaffolds. Originally, biodegradable synthetic scaffolds were developed to reinforce and maintain the mechanical strength of TEVGs. In the 1980s, Weinberg and Bell produced the first TEVG. It consisted of cultured bovine ECs, SMCs, and fibroblasts in a collagen gel and subsequently was shaped into tubes. However, this TEVG lacked sufficient mechanical strength and required a Dacron mesh support. Since then, various approaches have been investigated. Niklason et al. produced a novel approach using a biodegradable synthetic scaffold for cell culture. The synthetic scaffold was designed to degrade as ECMs obtain the appropriate mechanical strength during cell culture within a bioreactor. Therefore, the final TEVG product is composed only of ECMs formed by cells.

Extracellular Matrices Formed by Cell Culture and Synthetic Polymers

As described earlier, Niklason et al. reported a unique approach to fabricating TEVGs. In vitro−fabricated human decellularized TEVGs using allogenic human vascular SMCs have undergone a multicenter clinical trial [7]. In brief, human vascular SMCs were derived from deceased organ and tissue donors and met eligibility requirements set forth by US Food and Drug Administration regulations. SMCs were isolated with 1−60 million cells from individual donors. After expansion, 1 million cells/cm were seeded onto polyglycolic acid (PGA) polymer scaffolds within flexible bioreactors. Pulsatile flow was applied to the grafts for 8 weeks in the bioreactors, and then subsequently were decellularized (Fig. 58.2). The grafts were implanted into the arms of patients with ESRD for hemodialysis access and showed favorable results. Of the 60 patients with ESRD who underwent TEVG implantation, primary patency was 63% and 28% at 6 and 12 months, respectively, and secondary patency, which included preceding successful interventional or surgical procedures, was 97% and 89%, respectively. The patency was higher than that reported in multicenter studies of ePTFE (55−65% at 1 year). In addition, the grafts displayed no significant potential immunogenicity, aneurysm, or structural degradation. This TEVG will require longer follow-up observations in addition to prospective, randomized, and large clinical trials. However, this TEVG is perhaps the closest to being clinically translated and serving as a readily available off-the-shelf conduit in large-diameter graft applications. In this chapter, the TEVG developed by Niklason et al. is classified as a hybrid scaffold even though the

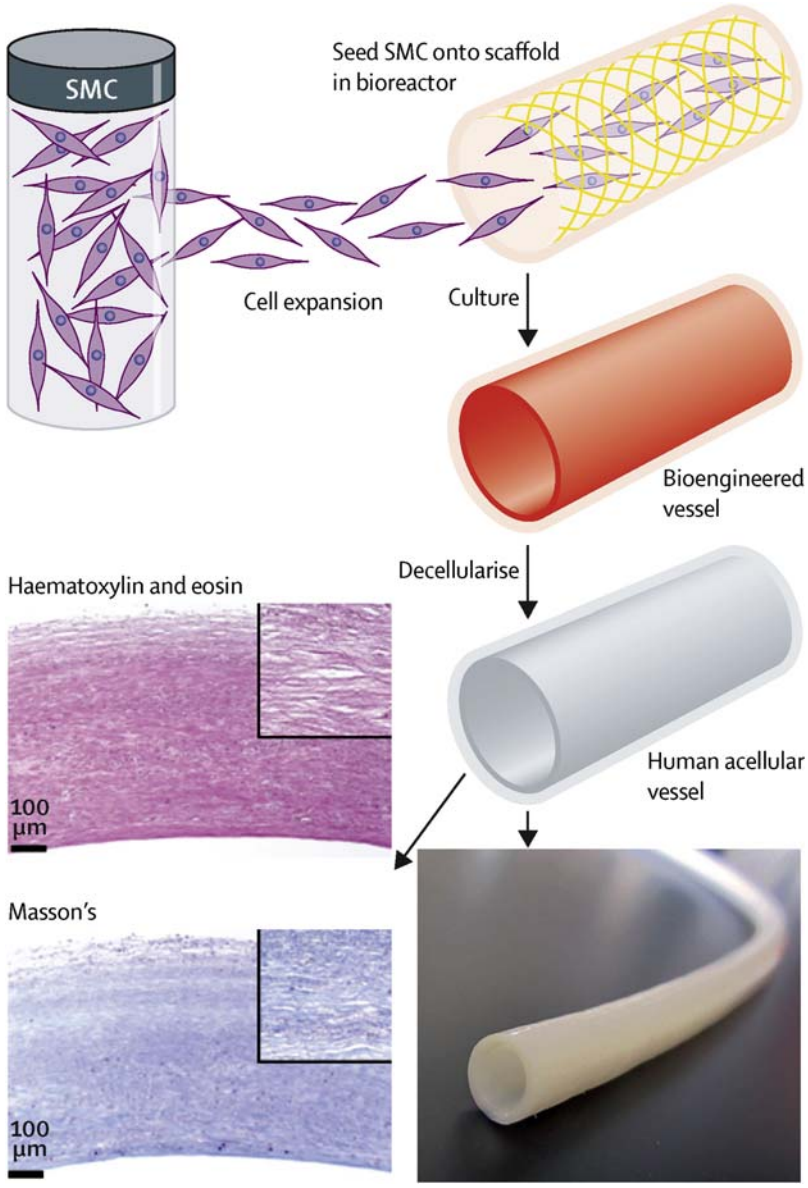

FIGURE 58.2 Production of arteriovenous shunt grafts by Niklason et al. Smooth muscle cells are seeded onto a biodegradable scaffold within a single-use bioreactor. During culture, a cellular bioengineered vessel is grown that is then decellularized to produce the human acellular vessel. Gross appearance is an off-white uniform tubular structure. Hematoxylin-eosin stain and Masson trichrome stain show a dense extracellular matrix without cellular or nuclear remnants. *SMC*, smooth muscle cell. *Lawson JH, Glickman MH, Ilzecki M, Jakimowicz T, Jaroszynski A, Peden EK, et al. Bioengineered human acellular vessels for dialysis access in patients with end-stage renal disease: two phase 2 single-arm trials. Lancet 2016;387(10032): 2026–34. Reprinted with permission from Elsevier.*

synthetic scaffold degraded before the grafts were implanted into patients. However, it is also reasonable to classify their graft as a biological-based scaffold.

Nature-Derived Polymers and Synthetic Polymers

The oldest hybrid TEVG applied clinically is Omniflow II, which is composed of cross-linked ovine collagen and a polyester mesh endoskeleton. Since the 1990s, several favorable study results have been published on this graft [30,31] and have led to its use in many European countries and several South American and Asian nations. Conceptually, a hybrid scaffold is one in which biological-based materials enhance biocompatibility and cell infiltration, whereas synthetic-based materials provide the graft with overall structure reinforcement. Many biological-based scaffolds have been investigated, including fibrin, collagen, elastin, chitosan, and gelatin. Synthetic-based scaffold approaches are primarily biodegradable. Poly(ε-caprolactone) (PCL) is well-used as a biodegradable synthetic-

based scaffold material and has been investigated in small-animal models [32,33]. Weiss et al. reported successful results with a bilayered scaffold composed of an inner layer of recombinant human tropoelastin and an outer layer of PCL in a rabbit model [32]. Another group demonstrated good results with an acellular hybrid vessel of PCL, polyurethane, and collagen in canine models [33]. In a large-animal model, Hibino et al. investigated an electrospun PCL−chitosan nanofiber TEVG as ovine carotid artery replacements [34]. The TEVG showed 67% patency (four of six) and there was no evidence of aneurysm or calcification. Electrospun nanofiber scaffolds are a promising method for fabricating arterial TEVGs that may be capable of withstanding high flow and pressure blood flow. Electrospun nanofiber scaffolds that resemble ECM structures can be fabricated relatively easily. In small-animal models, however, several studies revealed that an electrospun scaffold with small pore sizes can inhibit cell infiltration into TEVGs. Therefore, many researchers are searching for optimal scaffolds with regard to selecting the materials, electrospun fiber diameter, pore size, and alignment [35,36]. When blended into a TEVG, rapidly degrading chitosan is considered to promote better cell infiltration and rapid neovessel remodeling owing to pore size enlargement. Jockenhoevel et al. developed fibrin scaffold grafts encapsulated with autologous SMCs/fibroblasts and ECs. The construct was then reinforced with a poly(L/D)lactide 96/4 mesh. They implanted the TEVGs into sheep carotid arteries and observed them for up to 6 months [37]. Eighteen grafts ($n = 6$ for 1, 3, and 6 months' follow-up) were patent and there was one significant case of stenosis (3 months). There was no evidence of thrombus, aneurysm, or calcification. In addition, explanted grafts were observed to contain remodeled cellular distribution and mature autologous proteins. However, these TEVGs are associated with higher costs and longer production times owing to cell culturing compared with cell-free TEVGs. In addition, these carotid artery TEVGs diameters are about 4−5 mm and are not strictly included in large-diameter TEVGs.

Synthetic Polymers With Seeded Cells

This type of TEVG consists of a biodegradable synthetic polymer seeded with cells. A biodegradable synthetic scaffold must maintain the graft's mechanical properties until the new vessel is reconstituted by host-derived cells over the course of its inflammation-mediated process. Various cell populations such as SMCs, fibroblasts, and ECs have been cultured and seeded onto TEVGs to mimic the structure of native vessels. Stem cells have also been identified as a potential source for cell seeding because they have important roles in tissue regeneration, remodeling, and maturation. Several types of stem cells, such as embryonic stem cells (ES cells), induced pluripotent stem cells (iPS cells), and bone marrow−derived cells, have been seeded onto the biodegradable synthetic polymer for study. In the past, a TEVG seeded with ECs derived from ES cells was developed. However, research on human ES cells has been limited because of political and ethical concerns. On the other hand, iPS cells do not have these concerns, and opportunities in tissue engineering have been recognized for cell sheet TEVGs in mice inferior vena cava [38]. The TEVGs showed favorable patency, but seeded iPS cells primarily exerted a paracrine effect to induce neotissue formation and showed no direct change to vessel cells. The benefit to using iPS cells is that they do not require immunosuppressive therapy, but it is a complex process to fabricate TEVGs using this particular cell population.

The feasibility of using a tissue engineered pulmonary arterial graft with seeded vascular cells in an ovine model was demonstrated in 1997 [39]. The same group switched to using autologous bone marrow mononuclear cells (BM-MNCs) instead of vascular cells for clinical applications and developed a venous TEVG that consisted of a woven fabric made of a 50:50 mixture of poly(L-lactide and ε-caprolactone) (PLCL) reinforced with PGA. Previously, it was reported that multipotential cells existed in bone marrow and had the ability to differentiate into several cells types and organs in vivo [40]. In addition, several studies revealed that endothelial progenitor cells derived from bone marrow contributed to angiogenesis. However, Shinoka et al. revealed in a mouse model that the number of seeded BM-MNCs in the TEVGs decreased rapidly in the first few days after implantation and disappeared altogether within 1 week after implantation without differentiating into vascular cells [41]. BM-MNCs contain an abundance of cytokines that can enhance neovessel formation, and they likely act in a paracrine manner to recruit additional host cells during the remodeling process. With regard to BM-MNC harvest and seeding of Shinoka's TEVG, approximately 4−5 mL/kg body weight BM-MNCs were aspirated into a syringe from the anterior superior iliac spine of a patient with a puncture needle under general anesthesia. After several washing and concentration steps, BM-MNCs were seeded onto biodegradable polymer scaffold by pipetting. The outer surface of the seeded scaffolds was then sprayed with fibrin glue. In 2001, the group started a clinical trial implanting the TEVGs for extracardiac total cavopulmonary connections to 25 Japanese children. Their biodegradable TEVGs were thought to be particularly suitable in children because they have the potential to grow and remodel. Midterm (median, 16.7 months) and long-term results (mean, 5.8 years) revealed no evidence of aneurysm, graft rupture, graft infection, ectopic calcification, or graft-related mortality [42,43]. However, four patients died of graft-unrelated causes during the long-term follow-up and four required percutaneous angioplasty owing to stenosis. In one patient, the

TEVG was replaced with a PTFE patch 2 months after the original surgery because of unexpectedly slow tissue growth on the patch used for a lateral tunnel procedure. Histological findings of the explanted graft showed collagen-rich fibers and a surface covered with ECs [42]. Of 20 patients who were contacted for long-term follow-up, eight (40%) required no daily medications. Patients who are implanted with nonbiodegradable synthetic grafts such as ePTFE often require long-term anticoagulation and antiplatelet therapy. Shinoka's group is collecting long-term follow-up data (median, 13.0 years) (unpublished data); eight patients have died owing to graft-related causes and seven have undergone percutaneous angioplasty (seven have undergone percutaneous angioplasty (six balloon dilatation and one stent implantation)). This long-term mortality rate appears to be reasonable because most patients with congenital heart disease typically originally have severe conditions and are at high risk. Comparisons between postoperative and long-term follow-up angiography (11 years later) showed that the neovessels behaved and functioned like normal vessels without stenosis and aneurysm (Fig. 58.3).

The female patient presented in Fig. 58.3 was diagnosed with complex congenital heart disease including double outlet right ventricle, subaortic ventricular septal defect, small tricuspid valve and right ventricle, Juxtaposition of right atrial appendage, persistent left superior vena cava, and total anomalous pulmonary venous return. She underwent pulmonary artery banding at age 1 month and total cavopulmonary connection (TCPC) with enlargement of the pulmonary vein orifice using autologous pericardium at age 3 years. A 16-mm-diameter PLCL and PGA TEVG seeded with BM-MNCs was implanted between the inferior vena cava and pulmonary artery. Angiography after graft implantation showed that the TEVG was patent (Fig. 58.3, left, indicated by *arrows*). Furthermore, long-term follow-up (11 years later) angiography showed that the TEVG remained patent without aneurysm and displayed growth as the child grew older (Fig. 58.3, right, indicated by *arrows*). More recently, Shinoka et al. presented the first report of late-term (12 years) histological findings of our TEVGs [44]. This female patient was diagnosed with a single right ventricle, pulmonary stenosis, pulmonary stenosis, bilateral superior vena cava, a hemiazygos connection, and a common atrium with mild to moderate regurgitation from a common atrioventricular valve, and underwent TCPC with a common atrioventricular valve plasty when she was 4 years old. A 12-mm-diameter biodegradable synthetic TEVG seeded with BM-MNCs was implanted between the hepatic vein and pulmonary artery. Unfortunately, she died at age 16 years owing to multiple organ failure, low cardiac output syndrome, disseminated intravascular coagulation, pancytopenia, and brain abscessation. Histological examinations of ECs, SMCs, elastic fiber and collagen fiber layers from the patient's autopsy report revealed that the synthetic scaffold had completely degraded, remodeled, and generated neotissue that seamlessly integrated with native vessel (Fig. 58.4).

In the United States, Drs. Breuer and Shinoka started a pilot study investigating the clinical use of the TEVGs in congenital heart surgery (NCT01034007). If the pilot study proves to be successful, this TEVG technology will be the first to be commercially applied clinically in the world. Several studies have already shown excellent long-term

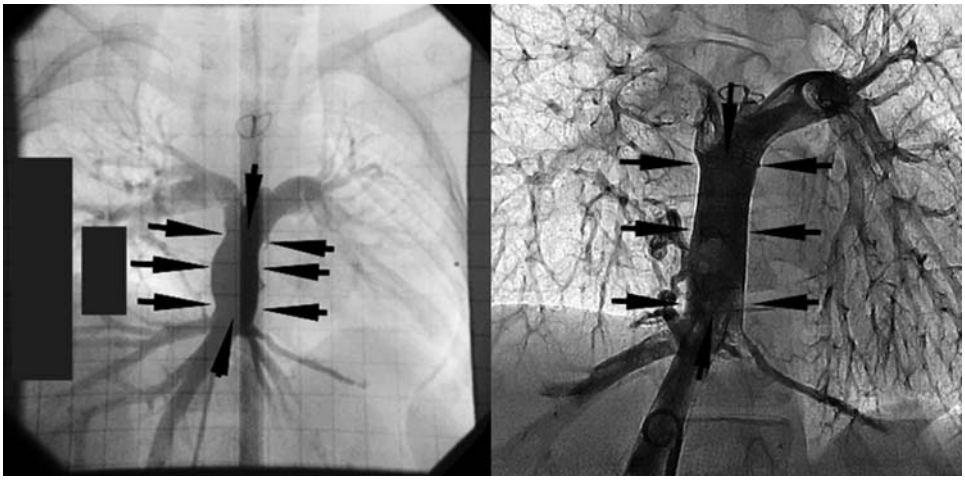

FIGURE 58.3 Angiographic comparison between postgraft implantation and long-term follow-up (11 years later). A 3-year-old girl with a double-outlet right ventricle, who underwent pulmonary artery banding at age 1 month, underwent a total cavopulmonary connection with enlargement of the pulmonary vein orifice using autologous pericardium. Poly(L-lactide and ε-caprolactone) (16 mm in diameter) and a polyglycolic acid tissue engineered vascular graft (TEVG) seeded with bone marrow mononuclear cells was implanted between the inferior vena cava and pulmonary artery. An angiography after graft implantation showed that the TEVG was patent (*left*) (*arrows*). Long-term follow-up angiography (11 years later) was patent with no aneurysm and displayed growth as the patient grew older (*right*) (*arrows*).

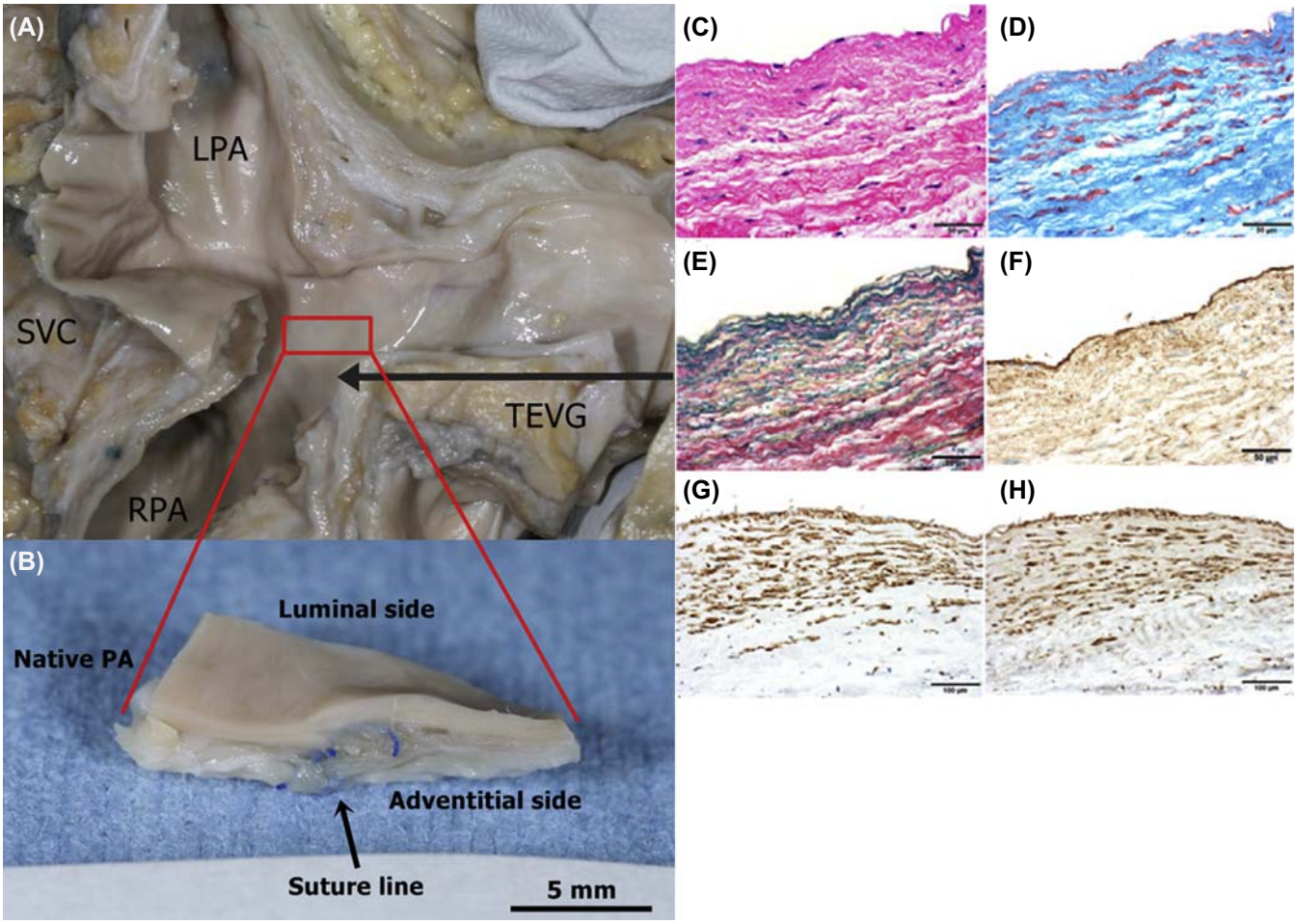

FIGURE 58.4 Macroscopic and histological images of explanted human TEVG. A female patient with a single right ventricle underwent TCPC with common atrioventricular valve plasty at age 4 years. A 12-mm-diameter biodegradable synthetic TEVG with BM-MNCs was implanted between the hepatic vein and pulmonary artery. Unfortunately, she died 12 years later and an autopsy was performed. (A) Macroscopic imaging of the TEVG. No difference appears between native PA and TEVG. (B) Cross-section view of the border between native PA and TEVG. (C) Hematoxylin-eosin staining. (D) Masson trichrome staining. (E) Victoria blue-van Gieson staining. (F) Factor VIII–positive cells (*brown*). (G) α-Smooth muscle actin–positive cells (*brown*). (H) Calponin-positive cells (*brown*). Bar length in C, D, E, and F are 50 μm, and bar length in G and H are 100 μm. *LPA*, left pulmonary artery; *PA*, pulmonary artery; *RPA*, right pulmonary artery; *SVC*, superior vena cava; *TCPC*, total cavopulmonary connection; *TEVG*, tissue engineered vascular graft. *Modified from Matsumura G, Shinoka T. First report of histological evaluation of human tissue-engineered vasculature. J Biotechnol Biomater 2015;5:200 under copyright.*

clinical outcome after TCPC using nonbiodegradable PTFE grafts [45,46]. This makes the superiority of biodegradable TEVGs much more difficult. Furthermore, the TEVGs used in TCPCs were implanted in high-flow and low-pressure environments. Therefore, the future will look at developing small-diameter TEVGs that are able to endure the high-flow and high-pressure environments of the arterial system.

Many researchers have investigated various combinations of scaffold materials, fabrication methods, seeded cells, and culture protocols. Beside the Breuer–Shinoka and Niklason groups, no clinical trials are using hybrid scaffolds. In ovine models, Hoerstrup et al. have shown patency of up to 100 weeks in pulmonary artery replacement with nonwoven PGA scaffolds coated with a thin layer of poly-4-hydroxybutyrate seeded with autologous myofibroblasts and ECs [47,48]. The scaffolds were fabricated by a heat-application welding technique. Thereafter, myofibroblasts were seeded in three steps at 24-h intervals onto the scaffolds and cultured under static culture conditions. After 2 days, the grafts were seeded with ECs. After another 3 days of static culture, the grafts were transferred into a pulse duplicator system and cultured for 14 days. Therefore, the total culture period is about 3 weeks and biodegradable synthetic materials remain in the grafts. This fabrication method is similar to that of Niklason et al., but there are major differences in the culture period and the fact that biodegradable synthetic materials remain when the graft is completed. Hoerstrup's hybrid TEVGs remodeled into a functional vasculature that mimicked native vessels and displayed growth as the sheep aged.

Biodegradable Synthetic-Based Scaffolds

The ideal TEVG is thought to remodel, generate neovessels, grow, and be easy to fabricate. With this in mind, TEVGs fabricated from acellular synthetic scaffolds are the closest approach to producing the ideal graft. Acellular synthetic scaffolds do not require in vitro cell cultures and can eliminate the risk for adverse immunological reactions. Therefore, cell-free biodegradable synthetic grafts have been investigated in small- and large-animal models. Poly(lactic acid), PGA, and PCL are the most widely used synthetic scaffold materials and have different degradation periods and mechanical properties. Attempting to combine the best various individual characteristics of single polymers, copolymers such as PLCL and poly(L-lactic-*co*-glycolide) have been proposed and developed. However, cell-free synthetic scaffolds are disadvantaged because they have no cell populations, like SMCs, fibroblasts, and ECs, to promote TEVG remodeling and antithrombogenicity. Therefore, acellular synthetic grafts have not yet reached clinical trials. Matsumura et al. have developed TEVGs consisting of knitted PGA fibers and a PLCL sponge with outer poly(glycolide-*co*-ε-caprolactone) monofilament reinforcement. These constructs were implanted into canine left pulmonary arteries and showed 100% patency up to 12 months [49]. This favorable patency is unsurprising because pulmonary artery reconstruction surgery generally results in satisfactory outcomes. The next generation of acellular synthetic scaffold will focus on arterial TEVGs studied in large-animal models. In arterial vasculature, lots of research has focused on small-diameter TEVGs because many patients have CVDs that require arterial bypass grafts or surgical procedures.

CONCLUSION

Historically, the development of TEVGs has faced many difficulties. To overcome these obstacles, different fabrication methods, techniques, and approaches have been proposed. TEVG materials introduced in this chapter are biologically or synthetic-based scaffolds. With regard to the development of TEVGs, it was rational to focus on the venous system at first, because large-diameter venous grafts do not need to endure the high pressures of the arterial system. In the 2000s, after Shinoka et al. published clinical results investigating the use of venous biodegradable synthetic grafts seeded with BM-MNCs in children with congenital heart disease, many researchers turned their attention to creating AV shunt TEVGs for hemodialysis access. This is the next logical progression in TEVG technologies because although the AV system has higher pressures than the venous system, it is still subject to lower pressures than the arterial system, which makes it much more likely and easy to fabricate. However, AV shunt TEVGs must be more durable than venous TEVGs and able to withstand repeated cannulation. Niklason et al. showed the viability of acellular biological-based TEVGs for patients with ESRD requiring dialysis access. The final frontier in vascular grafts will be to develop arterial TEVGs. Several research groups have developed arterial TEVGs and demonstrated their efficacy in animal models, but no groups have progressed to clinical trials. Most biodegradable synthetic arterial TEVGs are using electrospun nanofiber to withstand the high pressures of the arterial system. However, biodegradable synthetic TEVGs must be completely reconstituted by host-derived cells before the synthetic scaffold degrades and loses mechanical integrity. The neovessel remodeling process should include confluent endothelialization, the construction of SMC layers, and abundant ECM deposition. Therefore, the balance between scaffold degradation and neovessel formation has an important role in the long-term viability of a TEVG. Electrospun nanofibers are promising scaffold materials for arterial TEVGs. PCL electrospun nanofiber grafts have shown good mechanical properties with high patency rates in small-animal models [36]. However, thin-fiber and small-pore electrospun nanofiber scaffolds inhibit cell migration into the scaffold, which causes delayed neotissue formation. In contrast, thicker-fiber and large-pore electrospun grafts can enhance the neovessel formation and remodeling process by mediating macrophage polarization toward an M2 phenotype [36,50]. However, electrospun grafts with too-large pores can leak blood and lose their mechanical properties. Therefore, it is critically important to find the optimal blend of fiber diameter and pore size in electrospun nanofiber scaffolds when fabricating the ideal arterial TEVG. The clinical translation of small-diameter arterial TEVGs is greatly anticipated based on market forces and the many patients who have atherosclerotic CVD that require such grafts.

Important factors to consider when rationally designing a TEVG are that it should be less invasive, cost-effective, time saving, and readily available off-the-shelf. Unfortunately, venous and AV shunt TEVGs are not yet widely accepted in the clinical setting because not all of these factors have not yet been satisfied. Furthermore, the cell culture and seeding processes for producing TEVGs are technically complicated, and they have high costs and require extra time. With this in mind, cell-free synthetic TEVGs are the most cost- and time-effective approach but are limited by their inability to control cell migration, which often leads to aneurysm or occlusion. Therefore, technologies for TEVGs that elute and/or release drugs and proteins such as chemokines, cytokines, and growth

factors might be the source of important future studies and research. Several decades have passed since the first TEVG was developed. However, no one has developed a TEVG that is widely and commercially used in the clinical setting. To achieve clinical translation, multidisciplinary collaborations among clinicians, cardiovascular surgeons, engineers, chemists, and biologists will be needed in addition to further progress and advances in diverse technologies.

List of Acronyms and Abbreviations

AV Arteriovenous
BM-MNCs Bone marrow mononuclear cells
CABG Coronary artery bypass grafting
CAD Coronary artery disease
CVD Cardiovascular disease
EC Endothelial cells
ECM Extracellular matrix
ES cell Embryonic stem cell
ePTFE Expanded polytetrafluoroethylene
ESRD end-stage renal disease
iPS cell Induced pluripotent stem cell
PAD Peripheral artery diseases
PCL Poly(ε-caprolactone)
PGA Polyglycolic acid
PLCL Poly(L-lactide and ε-caprolactone)
PTFE Polytetrafluoroethylene
SIS Small intestinal submucosa
SMC Smooth muscle cells
TBTE Thread-based tissue engineering
TCPC Total cavopulmonary connection
TESA Tissue engineering by self-assembly
TEVG Tissue engineered vascular graft

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CHAPTER

59

Regenerative Medicine Approaches for Tissue Engineered Heart Valves

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INTRODUCTION

Heart Valve Disease: A Local and Global Problem

More than 5 million Americans are diagnosed with heart valve disease (HVD) each year. HVD can occur in any single valve or a combination of the four valves, but diseases of the aortic and mitral valves are the most common in adults [1–4]. Nearly 30% of all adults aged over 65 years have a sclerotic aortic valve, 10% of whom have accompanying stenosis (narrowing) [1–4]. Valvular heart disease is also a global issue. Approximately 1 million people aged over 65 years are affected by HVD in the United Kingdom [5]. US research also suggests that there could be undiagnosed HVD in the United States and United Kingdom populations [6]. Whereas the prevalence of valvular heart disease is estimated at 2.5% in industrialized countries, the prevalence in developing countries is unknown, but most likely it is much higher owing to the number of people (mostly children) with rheumatic heart disease (RHD). RHD is responsible for about 233,000 deaths annually [7,8]. At least 15.6 million people are estimated to be affected by RHD; a significant number of these are children and require repeated hospitalization and often unaffordable heart surgery over the ensuing 5–20 years [9–11]. In addition, RHD still represents 22% of valvular heart disease in Europe [7–11].

Adult Populations

As much as 90% of patients with severe aortic valve stenosis have a life expectancy of less than 10 years unless treated, and 50% of these patients with heart failure will die within 1 year [12]. The most common heart valves replaced in adults are the aortic and mitral valves; they represent three out of four cases of valvular disease in this cohort of patients [12,13]. Replacement of diseased heart valves by mechanical prostheses or bioprostheses is common and lifesaving and enhances quality of life in many patients. However, there are important limitations to available substitute heart valve technology, including (1) the need for long-term anticoagulation to reduce thromboembolic complications in all patients with mechanical prosthetic valves and the consequent significant risk of hemorrhagic complications with this therapy, and (2) progressive immune-based degradation of biological valves (including xenogenic and allogenic valves).

Young Populations

Congenital heart disease occurs in about 1% of births and more than one-third of these abnormalities involve the aortic or pulmonary valves. There is a significant need for valve replacement in children and adolescents [7–11]. A major problem with heart valve replacement in children is that mechanical or bioprosthetic devices are incapable of growth. Thus, children with congenital heart disease and other children who need valve replacement often require multiple valve replacement operations to implant successively larger valves to accommodate somatic growth.

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Indeed, difficulties associated with valve replacement in children have provided strong motivation to the tissue engineering approach to valves, the goal of which is to have a living and dynamic valve, with structure, function, and homeostatic and adaptive mechanisms as close as possible to those of the natural valve. Tissue prostheses are readily available and come in different ranges. However, they are unavailable in sizes smaller than 19 mm and hence are unsuitable for small children even if annular enlargement techniques were used.

CLINICAL OPTIONS

More than 100,000 heart valve surgeries are performed in the United States each year, and over 300,000 worldwide [1–4]. At a mean cost of $140,000 per procedure, this indicates an annual cost of $14 billion in the United States alone [1–4]. HVD surgery is the second most common cardiovascular procedure and is expected to triple (exceeding 1 million worldwide cases annually) by 2050 [3]. Because of its important hemodynamic position, a sclerotic aortic valve increases risk for death from other cardiovascular diseases (e.g., heart attack, stroke, and cardiomyopathy) by 50% [1–4]. The invention and implantation of prosthetic heart valves to treat patients with dysfunctional, diseased valves started in the 1950s [1–4]. Since then, a wide variety of valve prostheses have been developed and can be divided into mechanical or biological.

Mechanical

Several designs of mechanical heart valves have been developed over the years [14,15]. The most common mechanical valve is the bileaflet valve introduced in 1977 [15]. Mechanical valve prostheses are usually recommended for patients aged under 60 years, because these prostheses are durable with the potential to last over 20 years and often do not require replacement surgeries [16–18]. However, mechanical heart valves increase the risk for thromboembolism and require lifelong anticoagulation therapy, which exposes them to higher risks for bleeding and hemorrhages associated with this therapy [18]. For infants born with congenital valve defects, mechanical valves are not ideal because they do not come in sizes small enough (16–29 mm in diameter) for newborns and do not grow with the patient.

Biological

Biological tissue-based valve replacements were developed to eliminate the need for anticoagulation treatment in patients who have undergone valve surgeries. Since the late 1950s, many different types of biological valves have been created, ranging from valves coming from the patient's own body (autografts and autologous valves) to valves from human donors (homografts) and to valves from other animal species (xenografts). Advantages of biological valves are their excellent hemodynamic profile, lack of anticoagulation therapy, and ability to grow and integrate with the patient [19]. Since their production in 1965, xenografts have become the most frequently implanted biological valve [20]. Two common classes of xenografts include porcine aortic valves and bovine pericardial valves [14]. Although biological heart valves eliminate the need for anticoagulation therapy, they are not as durable as mechanical heart valves. Patients must undergo valve replacement surgery within 10–15 years, because biological valves degrade over time. Thus, biological heart valves are often recommended for patients aged more than 60–70 years [21]. Biological heart valves are available for pediatric patients, but they have a higher rate of degradation and do not grow, and thus require repeat procedures [22,23]. Advantages and disadvantages of mechanical and biological valves are depicted in Table 59.1.

TISSUE ENGINEERED HEART VALVES

Tissue engineered heart valves (TEHVs) can potentially address the shortcomings of current implants by providing an unlimited supply of heart valves in a variety of sizes. The general approach to producing TEHVs is to incorporate biomaterials with autologous cells that enable growth and biological integration. The ideal heart valve prosthesis should be antithrombogenic, biocompatible, durable, and resistant to calcification, and exhibit a physiological hemodynamic profile [24]. In addition, the valve's capability to integrate and grow with somatic growth would eliminate the need for multiple surgeries. The overall goal of TEHVs is to demonstrate excellent hemodynamics and match patient anatomy. At a cellular level, biomaterials used for the TEHV construct should promote cellular integration by enabling cell adhesion, proliferation, extracellular matrix (ECM) production, and remodeling. Most important, the scaffold

TABLE 59.1 Overview of Advantages and Disadvantages of Existing Heart Valve Implants

Implant	Advantages	Disadvantages	References
Mechanical	Durable	Lifelong anticoagulation	[16]
	Low reoperation rates	Risk for hemorrhage and embolism	[17]
		Does not grow	[16,18]
		Infection risk	[18]
Biological	"Natural" material	Limited supply for pediatrics	[18,19]
	Better hemodynamics	Less durable	[19]
	No anticoagulants	Immune-based deterioration	[18,19]

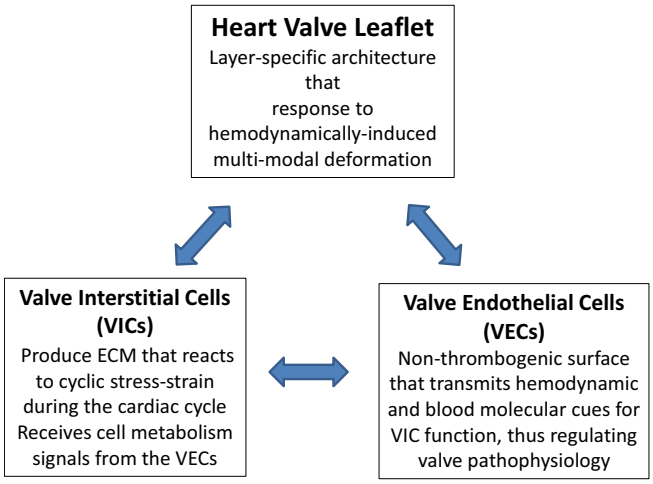

FIGURE 59.1 Schematic representation of the interaction of valvular endothelial cells (VECs) and valvular interstitial cells (VICs) on heart valve leaflet structure and function [25–28]. *ECM*, extracellular matrix.

must promote the physiological cell phenotype and degrade at a rate that matches ECM production. Despite advances, no currently available TEHVs meet all of these requirements. Thus, the challenge to bioengineering TEHVs is to develop commercially available implants that can meet all or most of these criteria. This will require the collaboration of biomechanical engineers, cell biologists, physiologists, pathologists, and cardiothoracic surgeons.

Biomaterials suitable for TEHVs fall into several classes: decellularized ECM (xenografts or homografts), natural polymers, and synthetic polymers. When designing a TEHV, there are three main design considerations for choosing a biomaterial: (1) cell–scaffold interactions, (2) the fabrication process and its impact on the microarchitecture of scaffold, and (3) the durability of structure and function in a physiological setting. This is especially important when designing valve replacements for the mitral or aortic position, in which pressures and shear stresses are much higher than the pulmonary position.

There are several design criteria for tissue engineering heart valve scaffolds. All of these need to focus on how best to recapitulate the basic structure of the valve leaflets. This consists of cellularization (either ex vivo or in situ) of a bilayer of valvular endothelial cells (VECs), surrounding valve interstitial cells (VICs), which have properties of both fibroblasts and myocytes (myofibroblasts). These cell interact both physically and biologically to form and maintain valve structure and function [25–28]. The basic biology of the heart valve leaflets is depicted in Fig. 59.1.

Considerations for Cell Source

There are several considerations for selecting a cell source. First, the type of cells is important to consider because there are three main options: xenogeneic, allogenic, and autologous. Although xenogenic sources may be readily available, they are not a viable clinical option. Allogenic cells are also not ideal because they can invoke immune

responses and would require long-term immunosuppressive therapy. However, allogenic sources may be possible if used to create stronger matrix, followed by decellularization and implantation. Thus, autologous human cells are the most desirable option for creating a TEHV.

The ideal cell source for seeding of TEHVs would be one that can be expanded sufficiently and has the potential to be differentiated into the two main cell types found in heart valves (VECs and VICs). If these cells are to be differentiated, it is important to characterize their phenotype and biological function [28]. VECs regulate permeability, immune and inflammatory responses, and VIC phenotype through paracrine signaling [27]. Meanwhile, VICs maintain tissue homeostasis by high proliferation activity and ECM repair and remodeling [27]. To recapitulate the long-term durability and function of a TEHV, it will be necessary to produce an implant that can reproduce these cell functions and spatial distribution within the valve leaflet.

A number of cell types have been applied to TEHVs. These include VICs, VECs, endothelial progenitor cells, bone marrow progenitor cells, autologous amniotic fluid cells, myofibroblasts, and smooth muscle cells [27]. The important takeaway from past studies is that each cell type has advantages and disadvantages. Although these studies are promising, the direct application of primary cells for TEHVs may not be suitable for clinical translation, because allogenic cells can induce an immune response in the recipient of the TEHV. Primary cells have limited proliferation capabilities whereas stem cells can result in uncontrollable proliferation and differentiation. These are some limitations that need to be considered when selecting a cell source for TEHVs.

Scaffold Design

The design of any implant will depend on the implant position within the heart and must recapitulate the leaflet structure. There are four heart valves: the tricuspid and mitral valve control blood flow from the atrium to the ventricles while the pulmonary and aortic valves regulate blood from the ventricles to the pulmonary artery and the aorta, respectively. The tricuspid, pulmonary, and aortic valve are composed of three leaflets. Meanwhile, the mitral valve has two cusps. Each leaflet is composed of three layers known as the fibrosa, spongiosa, and ventricularis. The fibrosa layer has collagen fibers aligned in the circumferential direction, whereas the ventricularis is composed of elastin fibers aligned in the radial direction. Valve endothelial cells line the outer layers of the fibrosa and the ventricularis, and within the valve leaflet, there are valvular interstitial cells [29].

Despite efforts, the ability to replicate the trilayer leaflet structure in TEHVs successfully remains challenging. One of the most important design criteria for developing functional leaflets is the different orientations of collagen and elastin fibers. To replicate this property, a fabrication process that can reproduce these unique orientations will need to be identified and implemented. Furthermore, the overall mechanical properties need to be within physiological parameters.

With newer fabrication techniques, several groups have developed and tested multilayer scaffolds for TEHVs. For instance, the electrospinning technique using materials such as poly-ε-caprolactone (PCL) enables the formation of aligned nanofibers [30]. These aligned scaffolds introduce anisotropic properties similar to native valve leaflets. In addition, modification to electrospun scaffolds and addition of other materials have since been introduced to generate more complex structures that resemble the three layers of the leaflet [31,32].

Once developed, consideration needs to be given to the interaction between layers of the valve implants. One study demonstrated that a trilayer hydrogel quasilaminate can be generated using poly(ethylene glycol) (PEG) diacrylate and a sandwich fabrication method [33]. The scaffold was composed of two stiff hydrogel outer layers and one soft hydrogel component to represent the spongiosa layer. The scaffold experienced no issues between the interfaces of the layers and did not vary in bending moduli compared with its single-layer counterpart. However, addition of cells to the trilayer scaffold reduced the modulus [33]. Three-dimensional (3D) printing also permits the fabrication of a leaflet structure that enables anisotropic properties to be introduced. However, 3D printing is still in the early development stage [33].

Implant Function

Proper and long-term implant function is the ultimate goal of valve replacement. In an average lifetime, a person's heart valves will open and close nearly 3 billion times, allowing 3–5 L of blood to circulate through the body continuously and efficiently [34,35]. The semilunar valves (pulmonary and aortic) prevent blood from flowing back into the ventricles, while the atrioventricular valves (tricuspid and mitral) prevent blood from flowing back into the atria from the ventricles. To sustain function, heart valves must be able to withstand many forces of the hemodynamic environment in both the right and left sides of the heart. Meeting these criteria is the challenge of TEHVs.

Hemodynamically, the leaflets of the aortic valve are closed during diastole, slightly overlapping in a region called the lunula to allow filling of the left ventricle. The valve annulus then expands in radius until a small orifice is present

and blood begins to flow through the orifice as the ventricle contracts during systole [34]. During this contraction, the aortic valve opens and blood flows through the valve, traveling up to 1.35 ± 0.35 m/s [36]. At the end of systole, the deceleration of blood flow allows for coaptation of the valve cusps [34,35]. As one can imagine, redeveloping these complex hemodynamic functions is a particularly difficult job using TEHVs, and further studies need to be performed.

The biomechanics of the implant are critical to valve hemodynamics. During the cardiac cycle, valves experience stresses and strains as a result of blood flow and pressure gradients between the chambers of the heart. Arjunon et al. described three mechanical stimuli experienced by heart valves: shear stress, pressure, and leaflet strain [25]. Shear stress in valves is defined as the frictional stresses that the leaflets experience owing to blood flow during the cardiac cycle. Strain is a measure of deformation. Strain in aortic valve leaflets is largely determined by tissue structure. Strains in the circumferential and radial directions were reported to be 10% and 40%, respectively [25]. The highest shear stresses experienced by the ventricularis are 70 dyn/cm^2 during systole; during diastole, the fibrosa experienced shear stresses of about 23 dyn/cm^2 [25].

Biomechanics and hemodynamics cannot be considered separately in implant design. In a diseased or defective state, leaflets may experience higher levels of shear stress owing to altered hemodynamics [25]. During diastole, heart valves experience transvalvular pressure gradients ranging from 10 to 120 mmHg [35]. The aortic valve, in particular, experiences a transvalvular diastolic pressure gradient of 80 mmHg [25]. The ability to sustain this pressure is caused by circumferentially aligned collagen fibers in the fibrosa layer of the leaflets [29,36]. Therefore, the hemodynamics and biomechanics are closely intertwined.

Implant Design Goals

TEHVs must be designed to offer a significant increase in function compared with the diseased valve while integrating with the patient's body, a task not yet accomplished by mechanical or biological valve implants. Above all, TEHVs must ensure long-term functionality of the valve by passively maintaining unidirectional blood flow. To achieve this functionality, TEHVs must be designed with several considerations in mind. They must be able to withstand the inertial forces of blood flow in the hemodynamic environment of the heart location in which they are implanted. Blood flow out of the left ventricle during systole should passively open the TEHV. By mimicking the general anatomy of the valve leaflets and sinuses, the pressure gradients between heart chambers should cause the TEHV to close fully. Continued and oscillating changes should not cause damage to the leaflets, nor should they stimulate the progression of disease, such as calcification. Finally, leaflets should display anisotropic properties, particularly regarding strain in the circumferential and radial directions, because these properties are important in ensuring valve functionality and preventing pathological conditions from developing on the cellular level [25].

Testing Tissue Engineered Heart Valve Function

There are two approaches for testing TEHVs: one is in vitro (before implantation or explantation) and the other is in vivo. Engelmayr et al. [37] used a bioreactor to simulate cyclic flexure to test heart valve scaffolds made of poly(glycolic acid) (PGA) fibers and a 50:50 blend of PGA and poly(lactic acid) (PLLA) fibers, coated in the β-subunit of prolyl 4-hydroxylase (P4HB) to allow molding into different shapes. From this study, they determined that dynamic flexure significantly decreases the stiffness of their scaffolds after 1 week compared with those under static conditions, owing to fiber fragmentation observed in scanning electron microscopy images of the scaffolds. This finding is significant in that dynamic testing is necessary to evaluate the functionality of TEHVs in a physiological setting, because TEHV behavior may not be truly represented under static testing conditions. Driessen et al. [38] measured the biomechanics of TEHV leaflets and created them in a diastolic pulse duplicator. Over 4 weeks, the conditioned leaflets became thinner. Uniaxial tensile tests were performed on the conditioned leaflets and the results were used to develop a model to analyze TEHV mechanics. In addition to less coaptation of the engineered scaffolds, these results indicated that the tissue engineered leaflets were stiffer and less anisotropic compared with native porcine valve leaflets These studies demonstrate the importance of testing the functionality of TEHVs under dynamic conditions and indicate a need for more appropriate scaffold designs to replicate the functionality of native valves.

Only a few in vivo studies involving TEHV implantation have been conducted. Sodian et al. created polyhydroxyoctanoate (PHO) scaffolds with ovine carotid arterial cells and implanted the scaffolds into six sheep. These valve scaffolds resulted in no stenosis or thrombosis for 20 weeks, but they degraded slowly, resulting in prolonged bioabsorption of PHO [39]. Hoerstrup et al. created a PGA mesh coated with P4HB, which resulted in faster degradation of the scaffold that was higher in strength and flexibility. These valve scaffolds resembled native heart valves in structure, mechanics, and composition, and they remained functional in sheep for 5 months [40]. More recently,

Kluin et al. implanted an electrospun polycarbonate *bis*-urea valve into the pulmonary position of sheep for up to 12 months. During that time, the subjects showed no signs of valve failure and the implanted scaffolds had cell infiltration into the porous microstructure. The evidence of collagen, glycosaminoglycans (GAGs), and elastin deposition similar to native valves was thought to contribute to the successful functionality of these valves [41]. Williams et al. [42]. transplanted porcine decellularized scaffolds, conjugated with CD133 into the pulmonary position of sheep and reported that these constructs had similar biomechanical and matrix composition as native leaflets 3 months after implantation. Furthermore, these CD133-conjugated valves could be cryogenically preserved and maintain their viability [43]. However, many TEHVs have yet to be studied for functionality. As more groups are successful in designing functional TEHV scaffolds, studies regarding the mechanical properties and the effect of hemodynamics on these scaffolds must be conducted to determine their suitability for implantation in humans.

Bioreactors

In vitro testing and conditioning of THEVs has required the development of bioreactors. Because hemodynamic behavior around valves is complex, many different bioreactors have been created to mimic the conditions that native valves experience, including cyclic stretch and flexure, oscillatory shear stress, and pressure. In 2000, Hoerstrup et al. developed a pulsatile flow bioreactor to stimulate TEHV scaffolds to increase in mechanical strength through ECM production while enabling a more complete degradation of the polymeric scaffold [44]. For less mechanically robust TEHVs made of materials such as hydrogels, Jockenhoevel et al. created a bioreactor consisting of a laminar flow chamber to introduce shear stress to fragile scaffolds [45]. Soon afterward, Engelmayr et al. reported a bioreactor design to provide cycle flexural stimulation to TEHV scaffolds to observe changes in effective stiffness of the scaffold resulting from unidirectional cyclic flexure [37]. To study the effects of conditioning scaffolds with only some of the mechanical cues present during the cardiac cycle, Mol et al. designed a diastolic pulse duplicator, a bioreactor that mimics only the diastolic phase of the cycle and conditions scaffolds using a strain-based approach [46]. Berry et al. and Lee et al. [47,48] developed a computerized pulsatile bioreactor to condition-seeded endothelial cells physiologically on a decellularized porcine scaffold. Alternatively, Engelmayr et al. designed the flex–stretch–flow bioreactor to study the coupled effects of cyclic flexure, stretch, and flow on TEHVs [49]. In addition, Syedain et al. created a cyclic stretch bioreactor consisting of the TEHV mounted in a latex tube that is cyclically pressurized by culture media, stretching the valve root and leaflets with the latex. More recently, Ramaswamy et al. designed a more physiologically relevant bioreactor that would allow for higher fluid velocities and subject scaffolds to higher shear stress while maintaining laminar flow [50]. From a regulation standpoint, bioreactor-required TEHVs represent a challenge because resterilization and reuse increases the risks of disease transmission between scaffolds during seeding and conditioning. This hinders the clinical development of patient-specific TEHVs, so alternative designs (disposable) must be pursued to make this process clinically translatable.

In Vivo Conditioning and Testing

Several approaches have been devised to rely on the body to reseed and condition implanted TEHVs, because the body is the best incubator and bioreactor. In vivo testing is difficult in small animals. For this reason, larger mammals are used, such as dogs, pigs, calves, and sheep, in which ultrasound, computed tomography, and magnetic resonance imaging of cardiodynamics are available. The most commonly used animal model for studying TEHVs is the adult sheep. Sheep have been widely used because they have a high calcium metabolism, which makes them valuable for observing TEHVs for durability and sustained performance under "worst-case" conditions [51]. Gallo et al. [52] reported on the potential use of the Vietnamese pig to evaluate TEHVs because they possess anatomic features that are similar to those of humans. Compared with common farm pigs, Vietnamese pigs are smaller and do not grow as rapidly; they reach about 70 kg during adulthood. Vietnamese pigs are also suitable because their cardiac output, stroke volume, mean arterial pressure, heart rate, and myocardial blood flow are similar to those of human beings.

BIOMATERIALS FOR TISSUE ENGINEERED HEART VALVES

Decellularized Bioscaffolds

One method for tissue engineering living, functional heart valves is to decellularize xenogeneic heart valves and then recellularize them with autologous cells with or without conditioning in a bioreactor before implantation. Several approaches to decellularization have been developed over the years. Each has advantages and

disadvantages. These include the use of detergents, enzymes, and osmotic buffers. Two commonly used decellularization detergents are sodium dodecyl sulfate (SDS), an anionic surfactant, and Triton X-100, a nonionic surfactant [53]. Enzymatic decellularization by trypsin−ethylenediaminetetraacetic acid is also commonly studied [53]. There are different protocols, which usually consist of removing the cellular material using one of these agents.

One problem with these decellarization procedures is structural changes within the scaffold biomaterials. Several studies have been conducted regarding the different methods of decellularization and their resulting impact on valve structure and mechanics. Decellularization using Triton X-100 has been shown to cause the largest increase of extensibility compared with treatment with trypsin or SDS [53]. However, these methods all had a significant effect on the stiffness of the leaflets. Treatment with SDS has also been shown to cause elastin fibers to curl, whereas collagen fibers lost structural details [54]. In this same study, trypsin decellularization resulted in decreased elastin and Triton X-100 caused collagen fibers to misalign. SDS was the only treatment that resulted in complete decellularization without the loss of elastin or collagen structure. A study reported that valves treated with SDS or Triton X-100 and trypsin did not show a significant decrease in elastin, but it resulted in a decrease in collagen and GAGs [55]. The results of these experiments failed to provide an optimal protocol for decellularization, which has yet to be developed. Recellularization of a decellularized scaffold involves coating the inner and outer surfaces of the valve with a cell solution [55]. After seeding, valves are preconditioned in a bioreactor to improve cell migration and proliferation, as well as cell alignment to flow conditions.

Natural Materials for Tissue Engineered Heart Valves

In addition to preformed decellularized scaffolds, there are numerous approaches using biomolecules to form TEHVs. Collagen is the most abundant protein in the body, with multiple genetic variants [56]. The most common type of collagen is collagen type I, which has been extensively studied in tissue engineering applications [57]. The other types of collagen are II, III, and V; these make up components of skin, muscle, bone cartilage, and tendons, respectively. Collagen can be fabricated into several different forms such as collagen sheets, sponges, hydrogels, pellets, or nanospheres. In the case of heart valve tissue engineering, collagen is high interesting mainly because the leaflets of the valve are primarily composed of collagen type I. Collagen can be extracted and purified from different tissue sources [58]. The mechanical properties of collagen hydrogels can be tuned through manipulations of the concentration of monomer, temperature, pH, and ionic strength [58,59]. These conditions can influence the diameter of collagen fibers and the overall mechanical properties of the hydrogel [60]. Although the mechanical properties are tunable, the elastic moduli of collagen hydrogels are limited to 10−30 kPa and have an ultimate tensile strength of 5−10 kPa [61]. However, these properties of collagen hydrogels are not representative of natural ECM because lower protein concentrations must be used to ensure proper diffusion of nutrients to the surrounding cells [34,62].

Heart valve collagen scaffolds have been molded in 2D or 3D hydrogels to better understand heart valve biology and the progression of valve disease [63,64]. To develop a scaffold with physiological properties, variations in the temperature, pH, and cross-link concentration have been used to modulate the scaffold properties [65−67]. These properties of collagen have been used to study the metabolic activity of VICs [68] and determine the effects of anisotropic strain on VIC phenotype and behavior [69].

There are both advantages and disadvantages to using collagen as a biomaterial for TEHVs. One advantage of collagen is that it is a native biomaterial with which cells normally interact [70]. One major disadvantage of collagen is variability depending on the source [71]. Another problem is that collagen from other species or even other humans can elicit an immunogenic reaction if antigens are present [58]. Importantly regarding TEHV use, collagen can activate the blood coagulation pathway through platelet adhesion and aggregation [72]. There are surface treatments to make the collagen less thrombogenic [73]. However, a major limitation is its mechanical properties; it must be combined with other materials for heart valve tissue engineering.

Fibrin

Fibrin is another natural biomaterial that has been used for TEHVs. Fibrin scaffolds support cell growth and proliferation and enable a number of biological active molecules to bind to fibrin [73]. This enables specific cell−matrix interactions that can promote tissue regeneration. Fibrin-based heart valves were created by molding [74]. In that study, Jockenhoevel et al. demonstrated that fibrin could be used to generate an autologous biodegradable tricuspid valve conduit using injection molding. The degradation rate of valve conduits was controlled by aprotinin, which halted fibrinolysis by inhibiting plasmin. Although injection molding of fibrin enabled a complex structure to be

made easily, there were two major limitations: scaffold shrinkage after long-term culture and poor mechanical properties. Another application of fibrin for TEHVs was when Flanagan et al. generated fibrin-based valve conduits, which were subsequently implanted into an adult sheep model for functional studies. The study used echocardiography to generate animal-specific valve models. The fibrin-based valves were generated by injection molding using a mixture of autologous fibrin, myofibroblasts, and endothelial cells [75]. Valves were conditioned mechanically for 28 days before implantation into the pulmonary trunk of adult sheep. Although the valves remained intact with no signs of thrombosis, calcification, or stenosis, valve insufficiency was observed in all implants owing to contraction of the leaflet structures [75].

There have been additional studies using fibrin-based TEHVs, but these involved hybrid constructs with fibrin and other biomaterials to address the issue of tissue shrinkage, contraction, and strength. The BioTexValve, developed by Moreira et al. is a bio-inspired textile-reinforced TEHV. The valve conduit is reinforced with electrospun fibers of poly(L/DL)-lactide and poly-lactide-*co*-glycolide (PLGA) to improve the leaflet burst strength; it closed with no problems under physiological aortic conditions in a flow-loop for 21 days [76]. Most fabrication of fibrin TEHV scaffolds has been limited to molding because fibrin cannot be manipulated after gelation and it does not have the shear-thinning properties for extrusion-based 3D printing [77]. The only method for 3D bioprinting fibrin is to use two-component printing, in which fibrinogen and thrombin are mixed on the print platform to form a hydrogel. Thus, fibrin alone is not suitable for 3D bioprinting, and it must be combined with other biomaterials for successful printing. Thus, fibrin can be used to make valve conduits that promote healthy cellular ECM production and remodeling without resulting in thrombosis or calcification. However, the major limitations of fibrin are the poor mechanical properties and scaffold contraction caused by cell-mediated contractions [73].

Alginate

This anionic polymer has been used in a wide range of biomedical applications owing to its biocompatibility, nonthrombogenicity, and low cost [78]. The overall mechanical and degradation properties of alginate hydrogels can be controlled via chemoreversible ionic gelation or covalent cross-linking [79,80]. Alginate hydrogels have been used in a variety of biomedical applications such as wound healing, drug delivery, and scaffolds for tissue engineering [71,78,80]. The main application for alginate in the area of TEHVs is to serve as a thickening agent for 3D bioprinting [30,81]. For instance, Duan et al. combined alginate with gelatin. This combination enabled valve conduits to be 3D printed with high shape fidelity and spatial placement of encapsulated cells [81].

There are many advantages and disadvantages to using alginate as a natural biomaterial for TEHVs. First, alginate is biocompatible, inexpensive, and easily implemented into the 3D bioprinting process [78]. A major drawback of using alginate is that there can be large batch-to-batch variation, and biosynthesis of alginate through bacteria is expensive and not yet scalable. Furthermore, its hydrophilic properties make it difficult for protein adsorption and cellular recognition. As a result, alginate scaffolds must be chemically modified to enable cellular function. Finally, there is concern that the presence of calcium ions can promote calcification of alginate scaffolds [80].

Hyaluronic Acid

Hyaluronic acid (HA) is a nonsulfated glycosaminoglycan that is a major constituent of the ECM. It is biosynthesized from bacteria (*Bacillus subtilis*) to produce highly pure human-grade batches [82,83]. Although the properties of HA can be used for tissue engineering, its poor mechanical properties require chemical modifications or the addition of other biomaterials to develop stable and durable scaffolds [84]. One application for HA scaffolds is to study VIC behavior under conditions mimicking the native valve [85]. The transition of HA into hybrid scaffolds permitted even more biological and mechanical control over the scaffolds. In addition, HA can be used to test a myofibroblast-like phenotype [86]. Thus, HA may be useful for understanding valve biology and pathogenesis. Skardal et al. demonstrated that HA can be used in conjunction with PEG derivatives to bioprint vessel-like structures [87]. They also generated a two-step bioprinting process with photocross-linkable hyaluronan and gelatin hydrogels [88]. HA lacks the mechanical properties necessary for physiological function as a scaffold for heart valve leaflets.

Gelatin

Gelatin is a partially hydrolyzed collagen that was originally investigated for its application as a cell culture platform. Gelatin has been incorporated into polymer blends to improve the viscosity for 3D extrusion-based printing and to introduce biologically active sites for enhanced cell viability [81]. The advantage of using gelatin is its similarity to collagen, in that it contains bioactive molecules that promote cell adhesion, proliferation, and migration. However, gelatin is mechanically weak and requires chemical modification or the addition of other materials for it to form a hydrogel. Thus, the best application of gelatin, like HA, is to introduce biological activity to a scaffold or to be used as an additive to modify the physical properties of a polymer blend for 3D bioprinting.

Chitosan

Chitosan is another natural biomaterial that has been applied to TEHVs. An interesting property of chitosan is that it is antimicrobial and has been investigated to treat biological prosthetic heart valves to make them antimicrobial and less prone to calcification [89]. An advantage of chitosan is its molecular structure, which is similar to GAGs. Furthermore, the biocompatibility and its ability to be degraded under physiological conditions are promising. However, chitosan is mechanically weak, similar to all other natural biomaterials, and thus must be modified or combined with other materials for its application in TEHVs.

Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) are a family of natural polyesters biosynthesized from microorganisms or modified plants, or through chemical reactions [90,91]. They are biocompatible, biodegradable, and tunable. They have been applied to the field of tissue engineering [92,93]. One of the first applications of PHA to TEHVs was the development of a composite valve conduit containing PGA. The scaffold was seeded with autologous ovine vascular and endothelial cells. Afterward, valves were implanted at the pulmonary valve position. The results of this study demonstrated that no thrombus formation occurred and there was evidence of cellular matrix production and remodeling [94] However, valvular regurgitation occurred after 24 weeks of implantation. Other studies include the use of PHA foam to generate a valve conduit seeded with autologous vascular cells [95]. The scaffolds were implanted into the pulmonary artery and after 17 weeks there was no sign of thrombus formation and minimal regurgitation compared with acellular scaffolds.

The benefit of PHA is that elasticity can be introduced to stiffer materials such as PGA or poly(lactic acid) (PLA). Previous studies combined chondroitin sulfate, HA, and bioactive glass to improve the bioactivity of PHA scaffolds [96–98]. However, issues that remain with PHA are shrinkage and valve regurgitation. This will likely require blending PHA with other biomaterials or introducing chemical modifications for a more useable scaffold. Table 59.2 outlines the most commonly used natural biomaterials in TEHV construction.

Synthetic Biomaterials

Because of their supply, consistent and reproducible content, and tunability, synthetic biomaterials have been pursued for TEHV fabrication.

Poly(ethylene glycol) Hydrogels

PEG hydrogels are used commonly for various tissue engineering applications because they are biocompatible, nonimmunogenic, and approved by the US Food and Drug Administration for internal use. They are also versatile, because their mechanical, structural, and degradation properties can be tuned using a variety of methods [73]. However, cells will not readily attach to PEG biomaterials because they are hydrophilic. Therefore, tissue engineering using PEG hydrogels requires the addition of bioactive molecules to the hydrogel, such as arginine-glycine-aspartate, a peptide found on fibronectin that promotes cell adhesion and migration [99]. Many studies have been conducted on the ability of PEG hydrogels to maintain the phenotype of native cells in heart valves as well as promote the production of ECM. One example is a study by Wang et al. [100], which demonstrated that VICs from porcine aortic valves maintain their quiescent fibroblast phenotype much better when cultured on PEG hydrogels as opposed to stiff plastics and suggested that the fibroblasts sense the elasticity of the surfaces by the phosphatidylinositol-4,5-bisphosphate 3-kinase–AKT pathway.

Several studies have also been conducted regarding the structure and functionality of PEG hydrogels in TEHVs. Durst et al. was the first group to study the bending mechanics of PEG hydrogels. They conducted three-point bending tests using a custom-designed bending tester on cell-seeded PEG hydrogels of different concentrations and molecular weights. The reported that the flexural stiffness of these constructs matched the native valve leaflets [101]. Other studies tested the importance the role of PEG in designing valve leaflet scaffolds that mimicked the trilayer structure of native leaflets [33], their use in 3D printing [102], and the importance of patterning PEG hydrogels in such a way that the anisotropic properties of native leaflets could be preserved [103].

Polyvinyl Alcohol

Polyvinyl alcohol (PVA) is a hydrophilic synthetic polymer generated by the hydrolysis of poly(vinyl acetate), which is formed by the polymerization of vinyl acetate [73]. This material has been pursued for tissue engineering

TABLE 59.2 Overview of Advantages and Disadvantages of Natural Biomaterial in Tissue Engineered Heart Valve Bioengineering

Biomaterials	Use in Heart Valve Engineering	Advantages	Disadvantages	References
NATURAL MATERIALS				
Collagen	Molded as hydrogels to study valve biology and disease progression, frequently combined with other materials	Most abundant protein in body Good cell interaction	Tensile strength Inconsistent batching Blood coagulation Immune reaction	[57,58] [70–73]
Fibrin	Used in molding heart valves and to test heart valve cell functions	Cell interactions Biodegradable Common natural protein	Poor mechanical properties and scaffold contraction caused by cell-mediated contractions Better combines with other biomaterials	[73–77]
Alginate	Thickening agent for three-dimensional bioprinting	Biocompatibility, nonthrombogenicity, and low cost	Large batch-to-batch variation Expensive to produce Hydrophilic properties make it difficult for protein adsorption and cellular recognition	[78–81]
Hyaluronic acid	Major constituent of extracellular matrix Study valve cell biology Bioprinting	Cell-friendly Used in bioprinting when mixed with other biomaterials	Poor mechanical properties	[82–87]
Chitosan	Treating biological prosthetic heart valves to make them antimicrobial and less prone to calcification	Biocompatibility and its ability to be degraded in physiological conditions are promising	Mechanically weak Must be modified or combined with other materials for its application in tissue engineered heart valves	[89]
Polyhydroxyalkanoates	Aids in introducing elasticity to stiffer materials such as poly(glycolic acid) or poly(lactic acid)	Biocompatible, biodegradable and tunable	Shrinkage and valve regurgitation	[92–98]

because it is nontoxic, biocompatible, biodegradable, and widely available [104]. In the development of TEHVs, PVA hydrogels have been studied for their cellular, structural, and functional compatibility. Wan et al. initially studied the fabrication of PVA hydrogels using the freezing and thawing technique to develop a bioprosthetic heart valve stent. They discovered that after the fourth freezing and thawing cycle, the stress–strain curve of the PVA hydrogel matched that of native porcine aortic roots in the physiological pressure range [105]. In other studies, PVA was used to attach fibronectin covalently to PVA to examine National Institutes of Health 3T3 fibroblast attachment and proliferation [106] to create photocross-linkable and degradable hydrogels [104]. A single-piece trileaflet valve made entirely of PVA molded using the freezing and thawing method was fabricated by Jiang et al. to develop a PVA-based composite hydrogel that mimicked the mechanical properties and anisotropic behaviors of native porcine aortic valve leaflets [107].

Hydrolytically Degradable Polymers

Hydrolytically biodegradable polymers such as PGA, PLA, PCL, poly(glycerol sebacate) (PGS), and polyurethanes (PUs) have shown promise for use in TEHVs because they are biocompatible and exhibit good mechanical properties suitable for withstanding the stresses and strains experienced by valves in a hemodynamic environment. Using techniques such as electrospinning, these polymers can be fabricated into fibrous scaffolds similar to the native valve ECM structure [92].

One of the most commonly studied biodegradable polymers is PGA. PGA has a high modulus and degrades rapidly, losing mechanical strength within 2–4 weeks; the accumulation of glycolic acid has been shown to cause inflammation months to years after implantation [92].

Another biodegradable polymer similar to PGA is PLA. PLA is more hydrophobic and stable against hydrolysis and it degrades more slowly compared with PGA. In tissue engineering applications, PLA has been blended or

copolymerized with other polymers to decrease degradation time [108]. PGA, PGA−PLA hybrids, and their copolymer PLGA have been used in a variety of studies regarding the fabrication of functional, bioresorbable leaflet scaffolds [109]. One group studied bone marrow stem cells seeded on PGA−PLLA scaffolds under different stress conditions and found that the cells exhibited endothelial and myofibroblast phenotypes similar to valves under combined steady flow and cyclic flexure [110]. Another group found that prolonged tissue culture reduced retraction of the tissue engineered leaflets that typically lose mechanical integrity owing to rapid degradation of the PGA scaffold, and suggested that this resulted from an increased amount of GAGs in the tissue [111].

PCL has a slower rate of degradation in vivo compared with PLA. As such, it has been used to contribute mechanical stiffness to tissue engineered valve leaflets. An early study that used PCL as a valve scaffold was conducted by Lieshout et al., in which a PCL filament was knitted into round leaflet patches that were sutured into a tube made of the same knitted PCL. The knitted structure was fitted on a mold and a fibrin solution was poured over the mold. The knitted scaffolds showed complete coaptation and durability over 10 million cycles. There was significant leakage, likely caused by areas of the scaffold where the fibrin detached [112]. In another study, seeded and unseeded scaffolds of PCL were fabricated and cultured for up to 6 weeks. From this study, the researchers determined that PCL scaffolds resulted in the most tissue formation over 6 weeks and the formation of tissue also increased the stiffness of the scaffolds. The tissue that formed on PCL scaffolds contained ECM components in amounts similar to those in native leaflets [113].

PGS is another biodegradable synthetic polymer that has been used to develop heart valve leaflets. The mechanical properties of PGS can be adjusted by altering the curing temperature, curing time, and molar ratio of glycerol to sebacic acid [114,115]. PGS undergoes surface degradation, slowly losing mechanical strength as a result of mass loss. Attempts have been made to create PGS scaffolds that mimic native valves in structure and function. Masoumi et al. designed and microfabricated PGS scaffolds by laser ablation, computationally determining the conditions necessary to create scaffolds that would best match the anisotropy and modulus of native bovine aortic valve leaflets. They observed moduli in the scaffold that were similar to the native valves in the circumferential and radial directions, as well as the presence of collagen and DNA in the scaffolds that were seeded with fibroblasts [116]. In a different study, Masoumi et al. used a micromolding technique to create trilayered scaffolds with PGS sandwiched between two layers of electrospun fibers of a PGS and PCL mixture. These trilayered scaffolds mimicked native valve leaflets in a number of ways, from promoting the deposition of ECM to mimicking their anisotropy and elastic moduli, and to displaying sufficient diastolic coaptation in the pulmonary position of pig hearts. However, the scaffolds lacked fibrous microstructure, which is a hindrance to tissue formation [31].

PUs are a class of elastomers that display good biocompatibility and tunable mechanical properties [117]. Some groups have worked to incorporate degradable linkages in the polymer to make it suitable for tissue engineering [108]. PUs have been used in TEHVs. Thierfelder et al. [118] fabricated PU scaffolds using a spraying technique and obtained homograft scaffolds, seeding both types of scaffolds with human fibroblasts and endothelial cells to compare their behavior after conditioning in a dynamic bioreactor for 5 days. Compared with the homografts, they found that PU scaffolds showed continuous, confluent layers of fibroblasts and endothelial cells on their surfaces, as well as decreased expression of genes associated with inflammation, interleukin-6 and monocyte chemoattractant protein 1, and the production of ECM. Table 59.3 outlines the most commonly used synthetic biomaterials used to construct TEHVs.

These are the biomaterials most commonly used to fabricate TEHVs. They all have advantages and disadvantages. The advantage of decellularized scaffolds is the premade 3D structure and "natural components." However, they have been shown to elicit an immune reaction and are limited in their supply and sizes. The advantages of synthetic polymers are their unlimited supply, consistent components, off-the-shelf possibilities, and ability to be made into multiple sizes and shapes. However, controlled degradation and tensile strength continue to plague their clinical application.

Tissue Engineered Heart Valve Fabrication Techniques

There are many techniques for fabricating natural or synthetic scaffolds. Scaffolds can be fabricated through molding, which is a process in which precursor solution is dissolved, poured into a mold, and then gelled. This technique is often used to generate tubular or planar scaffolds [58]. Another method for generating scaffolds is electrospinning. This fabrication process involves subjecting a polymer solution to an electric field. A charged polymer solution is generated when the electrostatic repulsion overcomes the surface tension between the polymer and syringe. Once this occurs, jets of polymer travel toward a surface, during which the solvent evaporates to generate continuous filaments of a natural or synthetic material [119]. Bioprinting is a more recent approach used to generate

TABLE 59.3 Overview of Advantages and Disadvantages of Synthetic Biomaterial in Tissue Engineered Heart Valve Bioengineering

Biomaterials	Use in Heart Valve Engineering	Advantages	Disadvantages	References
SYNTHETIC MATERIALS				
PEG	Used to maintain phenotype of native cells in heart valves, as well as promote production of extracellular matrix Used to modify flexural properties of implants	Biocompatible, nonimmunogenic, and approved by US Food and Drug Administration for internal use Versatile because as their mechanical, structural, and degradation properties can be tuned using a variety of methods	Cells will not readily attach to PEG biomaterials because they are hydrophilic. Therefore, tissue engineering using PEG hydrogels requires the addition of bioactive molecules	[73,99–103]
Polyvinyl alcohol	Used to develop bioprosthetic heart valve stents Used to create photocross-linkable and degradable hydrogels. Freeze–thaw capabilities	Nontoxic, biocompatible, biodegradable, and widely available	Poor strength on own. Must be used in conjunction with other biomaterials	[104–107]
Poly(glycolic acid) PLA	Functional, bioresorbable leaflet scaffolds. Behavior of cells on stiff biomaterials	Bioresorbable Made into many shapes and sizes	Fast biodegradation rates	[109–111]
Poly-ε-caprolactone	Scaffolds with slower degradation Add mechanical stiffness	Slower rate of degradation in vivo compared with PLA	Can make scaffolds too stiff and nondegradable unless tested in vivo	[111–113]
PGS	To adjust biomechanical properties of bioengineered heart valve leaflets	Biodegradable Maintains flexure properties similar to native valves	PGS-based scaffolds can lack fibrous microstructure and hindrance to tissue formation	[31,116]
Polyurethanes	Fabricate scaffolds using spraying technique and obtained homograft scaffolds, seeding both types of scaffolds with fibroblasts and endothelial cells	Good biocompatibility and tunable mechanical properties	Used only as addition to other biomaterials	[117,118]

PEG, polyethylene glycol; *PGS*, poly(glycerol sebacate); *PLA*, poly(lactic acid); *PEG*, poly(ethylene glycol); *TEHV*, tissue engineered heart valves.

scaffolds for heart valve tissue engineering. 3D bioprinting is an additive manufacturing technique that enables complex 3D structures to be generated through layer-by-layer deposition of biomaterials, cells, and growth factors [120,121]. The most common types of 3D bioprinting are inkjet, extrusion-based, and laser-assisted printing.

Future Direction in Tissue Engineered Heart Valves

Although the field has progressed significantly, no TEHV is being used clinically. As highlighted in this review, different cell types, recellularization methodologies, and biomaterials have been researched each with its own advantages and disadvantages. Fabrication techniques have evolved to develop more complex structures closely resembling native valves, but a main takeaway point is that a single biomaterial, cell type, or cellularization method may not be sufficient for TEHVs. The combination of several biomaterials and fabrication techniques may be the solution to addressing the limitations of using a single biomaterial. There are also the overriding problems of cost and patient accessibly to this complex bioengineering process. As reviewed in the Introduction, many people around the world do not have access to advanced bioengineering, surgical, or advanced cell processing facilities.

To address surgical facility limitations, transcatheter deployment of heart valves has become increasingly popular owing to its relative ease of implantation. Transcatheter valve prostheses consist of tissue mounted on an expandable stent; they are introduced into the body in a variety of ways, such as via the femoral artery, the aorta, or the subclavian artery, and transapically. All transcatheter valves are composed of biological tissue because this is necessary for the leaflets to be crimped into a catheter. Newer designs are being developed (Stephan and Petra). Regardless, there are concerns that the crimping may cause damage to the tissue and possibly affect long-term durability [122,123]. As a less invasive alternative to heart valve replacement surgery, transcatheter valve replacement allows patients who pose a high surgical risk to receive valve replacements they would not otherwise receive. Despite advances in the transcatheter delivery mechanism, stroke remains the major complication for transcatheter valve replacement

[124]. Although over 50,000 adult patients have received transcatheter valve implants since 2002, transcatheter technologies for pediatric patients with congenital heart disease are severely lacking [125]. However, the minimal invasiveness and fast recovery time of transcatheter valve replacement can be advantageous for younger patients, especially because valve-in-valve procedures are an option to replace damaged or degraded valve prostheses [124].

To make TEHVs more readily accessible to a wider patient population, an "off-the-shelf" approach may be needed. This involves a scaffold with the ability to remodel and form new tissue in the body through its recruitment of endogenous cells. This approach is advantageous over the traditional in vitro approach because it does not require preconditioning of the scaffold before implantation, which can take up to 6 weeks. During this preconditioning time, there are risks of bacterial and fungal contamination. The logistics of maintaining good manufacturing practice and infrastructure for translating an in vitro scaffold comes with an enormous cost [126].

One tested method of developing off-the-shelf TEHVs involves in situ heart valve scaffolds formed through foreign body reaction and decellularized TEHVs. Kishimoto et al. [127] developed an autologous heart valve scaffold using in-body tissue architecture technology. This involved placing a mold into the subcutaneous space, where the body forms a fibrous scaffold surrounding the mold. The stentless mold was tested in a goat model, where the valve performed well under systemic circulation. This approach enables off-the-shelf scaffolds to be created; however, it is in the initial stages of preclinical animal work. More assessment of the immune reaction to a foreign scaffold such as this is needed along with its long-term performance under physiological conditions.

Another approach involved first creating a biodegradable synthetic scaffold seeded with homologous cells. Then scaffolds were subjected to decellularization, in which the process is gentle enough not to alter the collagen or tissue structure. These researchers demonstrated that decellularized TEHVs could be stored for up to 18 months with no alterations to the tissue structure [128]. In addition, these off-the-shelf scaffolds have the ability to be reseeded with stem cells and maintain good cell viability through the crimping process for minimally invasive procedures [129]. Researchers have also demonstrated the feasibility and long-term functionality of homologous off-the-shelf TEHVs in a sheep and nonhuman primate model [130,131]. The concept of off-the-shelf TEHVs may be the fastest method to clinical application. Although this approach is promising, more research is needed for the recellularization, remodeling, and growth potential of off-the-shelf scaffolds. Concerns regarding thrombogenicity and calcification in the long term will also need to be assessed.

Other research groups have used hybrid tissue engineering approaches to develop off-the-shelf scaffolds with biological and mechanical properties capable of active remodeling after implantation. Janhavi et al. developed a biohybrid valve using electrospinning to create aligned nanofibrous scaffolds composed of decellularized bovine pericardium and PCL−chitosan. The scaffold was optimized to mimic the anisotropic and microstructure properties of native valves. The biohybrid scaffold was 20 times stronger than the native valve and demonstrated ECM deposition and cell proliferation when seeded with human VICs [132]. Although promising, this hybrid TEHV was not evaluated under physiological conditions.

Other investigators have taken the approach of developing a decellularized scaffold implant with alterations in surface characteristics that promote in situ recellularization and valve maturation [42]. This study decellularized porcine heart valves that were conjugated with the endothelial progenitor marker CD133. When placed in the pulmonary position of sheep, these constructs rapidly recellularized with both VECs and VICs. The conjugated valves displayed native valve-like biomechanical properties and interstitial matrix composition. Conjugated valves could be cryopreserved and maintained their recellularization properties when thawed [43]. These valves also displayed characteristics of growth that may make then desirable for pediatric patients [42]. However, they were tested only in the pulmonary position and for 3 months. It is unknown whether the implant can withstand the pressures and sheer of the eft side of the heart and if they continue to mature long-term.

Several studies have used modified biomaterials to attract cells for better regeneration and function. These include employing nanoparticles for a variety of uses [133,134]. Alternations in fibrospinning and coating strategies have also been used to attract cells to biomaterials [135,136]. Table 59.4 outlines the tested TEHVs and their advantages and disadvantages.

CONCLUSIONS

This review outlines the progress in and challenges of developing a clinically available TEHV. As discussed, there are several limitations to each option. These setbacks are particularly detrimental to younger patients who require a living valve with the ability to grow. Thus, the need remains for TEHVs. This review covered the design considerations for heart valve tissue engineering and assessed the application of biomaterials, fabricating techniques,

TABLE 59.4 Status of Current TEHV Constructs

Current TEHV Constructs	Advantages	Disadvantages	Status
Seeded (preseeded) natural biomaterial TEHVs	Three-dimensional architecture Cell compatible Natural hemodynamics	Immune response, degradation Lengthy, complicated, and expensive Cell viability can be low Do not grow Limited availability and sizes	Preclinical testing
Self-, or enhanced-seeding natural biomaterial TEHVs	Less time, cheaper, more off-the-shelf capabilities. This uses nanotechnology, biomolecules to alter characteristics	Unclear whether mobilized cells form durable growing valves	Preclinical testing
Synthetic biomaterial-based TEHVs	Multiple sizes, shapes and supply Less immunogenic Controlled degradation rates	Strength and durability	Preclinical testing
Seeded synthetic biomaterial-based TEHVs	Enhanced maturation Can use nanotechnology or biomaterial combinations to increase cell survivability and biomechanics	Strength and durability	Preclinical testing
Textile-reinforced TEHVs	Increase strength and durability Can incorporate cell seeding	Possible reduced degradation Possible reduced remodeling capabilities	Preclinical testing
Stent-delivered TEHVs	Wider clinical application Potential to enhance reseeding Less traumatic	Mitral valve still difficult to reproduce Postoperative slipping	Clinical application of unseeded implants, especially in Europe

TEHV, tissue engineered heart valves.

and emerging technologies for developing TEHVs. Despite enormous progress, a clinically available TEHV remains to be realized. A large class of natural and synthetic biomaterials has been investigated for TEHVs. However, as researchers have learned, using a single biomaterial may not be sufficient to match the demanding mechanical and biological properties of native valves. It seems that important progress has been made in developing biomaterials that could be off-the-shelf and stimulate self-reseeding and maturation into implants with long-term function and structure. It will be critical to develop a TEHV that has the capability to self-heal and remodel with growth. This is the challenge to address unmet medical needs.

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CHAPTER

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Regenerative Medicine of the Respiratory Tract

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Rebuilding or regenerating the respiratory tract requires a combined understanding of lung development, essential repair mechanisms, and the role of the supporting microenvironment. The primary function of the lung is to exchange gas to deliver oxygen to the circulating blood and remove carbon dioxide from it. Maintenance or reestablishment of this function is the fundamental goal of pulmonary tissue engineering and regenerative medicine. Although a seemingly simplistic job, many tightly controlled mechanisms are essential to permit and sustain this essential physiologic capacity. This chapter will review the lung's endogenous mechanisms for repair, as well as state-of-the-art approaches to harnessing this capability for tissue engineering and regenerative medicine of the respiratory tract.

LUNG DEVELOPMENT: A ROAD MAP TO REGENERATION

During development, the lungs, in addition to the trachea, arise from the anterior foregut endoderm, a tissue that ultimately leads to the creation of multiple organs, including the respiratory system, esophagus, thyroid, and liver [1]. In mice, lung specification is first noted at embryonic day 9 (E9.0) and is identified by expression of the transcription factor Nkx2.1 (Fig. 60.1). The primordial lung begins to form during the embryonic stage (E9.5–E12.5; weeks 4–7 in humans) and the original lung bud then splits into two bronchial buds. Separation of the posterior part of the trachea from esophagus also occurs. The process of branching morphogenesis occurs during the pseudoglandular stage (E12.5–16.5, weeks 7–17 in humans). This intricate process results in controlled development of the conducting airways and terminal bronchioles. The fibroblast growth factor (FGF) family of signaling proteins is an important driving force during development. Interactions with the surrounding mesenchyme have an essential role during airway morphogenesis, acting as the source of many important growth factors and promoting epithelial proliferation in the branching bud [2]. Lung branching can be partly recapitulated when the mesenchyme is removed but substituted with critical growth factors, including FGF10 [3]. During the canalicular stage (E16.5–17.5, weeks 17–26 in humans) and saccular stage (E18.5 to birth, weeks 27–36 in humans), the distal lung parenchyma develops, with the emergence of alveolar pneumocytes and the creation of the respiratory units of the lung. The glucocorticoid receptor is expressed during lung development and functions to stimulate the production of surfactant-associated proteins and phospholipid synthesis by enhancing the activity of phosphatidylcholine [4]. Surfactant production can be measured in the amniotic fluid during the saccular stage; it also prepares the lungs for breathing by reducing the surface tension of the alveolar unit. This milestone has clinical relevance because the ratio of the surfactant glycoproteins lecithin and sphingomyelin measured in the amniotic fluid can be used to indicate lung maturity, with rising lecithin levels correlating with gestational age [5]. As the understanding of pulmonary surfactant and its essential function evolved, the development of surfactant replacement therapy has greatly improved survival for preterm infants with immature lung function [6]. Lung development continues in the late and postnatal periods as the lungs undergo the process of alveolarization. This mechanism functions to increase the gas exchange surface area dramatically as the lung further refines the immature alveolar structure and undertakes secondary septation to generate a greater number of smaller alveoli [7]. The distal microvascular network also develops at this time to ensure that each alveolar unit is connected to the requisite blood supply. Postnatal lung development and alveolar remodeling continue through the first years of human life. This is coupled with

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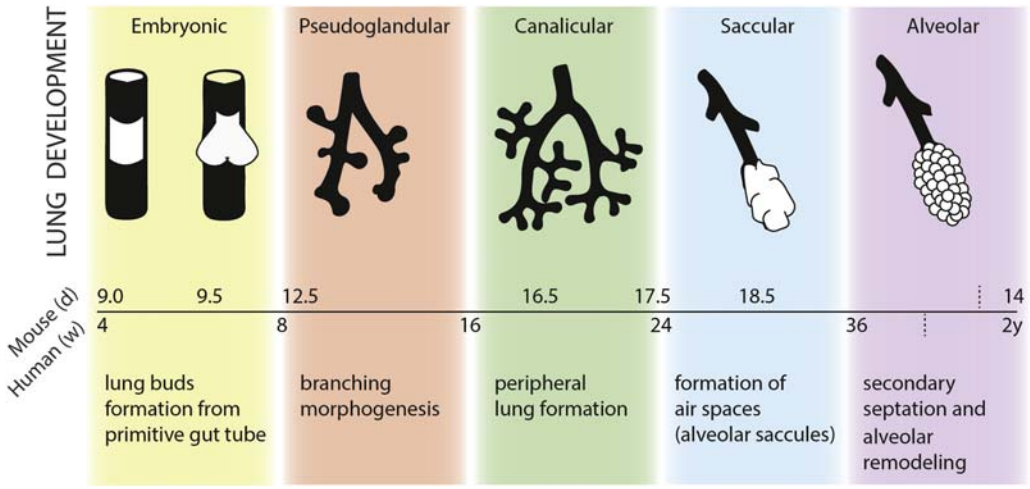

FIGURE 60.1 **Stages of lung development.** A timeline of lung development in the mouse (days) and human (weeks). Formation of the lung through defined stages, from early specification to birth (*dashed line*), and postnatal alveolarization, with key milestones listed.

normal tissue growth mechanisms to increase the functional capacity of the mature lung further. Morphometric data suggested that between birth and young adulthood, the total lung volume can increase 23-fold [8].

An understanding of lung development and the nuanced signaling pathways that dictate its successful manifestation provides an important foundation for appreciating the closely related processes of tissue repair and regeneration.

REPAIR AND REGENERATION IN THE NATIVE LUNG

Although adult lung tissue is often described as being largely quiescent, a remarkable capacity for repair remains after development is complete. As an organ that is constantly subjected to injury from the outside environment, a number of facultative repair mechanisms exist, which together act to maintain lung function and homeostasis. Furthermore, a healthy airway epithelium provides an important first line of defense against inhaled toxins and microbes, relying heavily on innate immune mechanisms to prevent pathogen colonization and mediate repair [9]. Maintenance of the lung epithelial barrier is accomplished by a number of regenerative cell populations that exist in specialized niches and can become activated after tissue injury.

Cell phenotypes that line the respiratory tract vary along a proximodistal axis. The bronchial epithelium largely functions to clear mucous and protect against immediate insult or prolonged inflammation. A continuous intact cell barrier is essential for this defense against environmental infiltrates and infections. The trachea and bronchi are lined with pseudostratified columnar epithelium consisting of ciliated, club, basal, and abundant goblet cells. The mucous secreted by goblet cells is a complex mixture of water, glycoproteins, and lipids designed to trap pollutants and aid clearance [10]. The accompanying ciliated cells are terminally differentiated columnar epithelium with coordinated cilia that beat to move the viscous mucous upward toward the pharynx, maintaining airway balance. The bronchial epithelium also functions to release cytokines, chemokines, and other mediators to recruit inflammatory cells and initiate host defense pathways when necessary. The secretory club cells are less abundant in the larger airways and account for 15% of proliferating cells during normal tissue homeostasis [11]. Club cells are further responsible for metabolizing inhaled toxins, which is accomplished by the cytochrome P450 isoform 2F within their smooth endoplasmic reticulum, which also makes them susceptible to ablation by treatment with the drug naphthalene [12]. Their major secreted product, the club cell secretory protein, 10 kD (CC10) (also known as secretoglobulin 1A1 and uteroglobulin) is also reported to possess antiinflammatory and immunomodulatory properties [13] and can be used as a noninvasive indicator of acute or chronic lung injury [14]. The basal epithelial cell lies superficial to the basal lamina and functions to aid attachment of the epithelium to the basement membrane. Basal cells can be identified by expression of transformation-related protein 63 (p63), cytokeratins 5 and 14, and the epidermal growth factor receptor in both mice and human airways [15]. Basal cells possess the ability to act as an endogenous adult stem cell population; they are capable of self-renewal and multilineage differentiation. Early experiments showed

that basal cells can function to reconstitute a differentiated mucociliary epithelium when seeded onto denuded tracheas and transplanted subcutaneously into nude rats [16]. Lineage tracing experiments further confirmed that basal cells can differentiate to club and ciliated cells in the trachea, both in steady state and after injury [17]. Loss of the basal stem cell population prevents proper epithelial tissue repair, resulting in pathologies similar to bronchiolitis obliterans syndrome, with alternating regions of epithelial denudation and progressive collagen deposition [18]. Similarly, impaired basal cell reparative function may contribute to the pathogenesis of chronic obstructive pulmonary disease in smokers [19]. Mathematical models suggest that a heterogeneous population of basal stem cells exists within the lung tissue and propose that approximately equal numbers are multipotent stem cells and committed precursors [20]. There is also evidence that rare CC10-positive cells can differentiate back to a basal cell lineage after tracheal injury in rats [21].

As the conducting airway branches toward the respiratory bronchioles, the cell composition progressively changes. The simple columnar ciliated epithelium of the terminal bronchioles develops into simple cuboidal epithelium. The smaller airways and bronchioles consist of basal, club, and ciliated cells, whereas goblet cells are no longer found and neuroendocrine cells appear. Neuroepithelial bodies (NEBs) appear, which have been shown to sequester regenerative cells capable of repairing the bronchiolar epithelium after selective depletion of club cells by the toxin naphthalene [22]. A subpopulation of cells within the NEB that are resistant to naphthalene, termed variant club cells, are also critical for preservation of this stem cell population and maintenance of epithelial renewal after injury [23].

The simple squamous epithelium of the distal lung consists of the flat, elongated alveolar type 1 pneumocytes (AT1), contributing 95% of the gas-exchange surface area, and the cuboidal alveolar type 2 pneumocytes (AT2), comprising the remaining 5%. AT2 cells are responsible for producing the pulmonary surfactant proteins (SP) A through D, whereas the AT1 cells interface with the pulmonary vasculature to facilitate gas exchange. Early experiments first provided evidence that AT2 cells serve as endogenous progenitors for the distal lung, which become type 1 cells in response to injury [24]. This observation was further confirmed using more sophisticated genetic lineage tracing techniques, demonstrating the clonal proliferation of AT2 cells during long-term homeostasis and after bleomycin injury [25]. A distinct population of cells at the bronchoalveolar duct junction has been described to have unique regenerative properties, with a bilineage potential for repair. These bronchioalveolar stem cells are reported to coexpress CC10 and SP-C and to proliferate in response to epithelial damage in vitro [26]. In vivo, lineage-tracing studies found that CC10-positive cells did not significantly contribute to the alveolar epithelium during postnatal growth [21]. After bleomycin injury, CC10-labeled SP-C−negative cells have been shown to develop into AT2 cells, combined with self-renewal of the native AT2 population [27]. Together these studies continue to redefine the multilineage potential of traditionally proximal or distally restricted cell populations and to be an important and expanding area of research. It has been shown that during development, the distal AT1 and AT2 cells arise from a bipotent progenitor, in contrast to the traditional hierarchy shown after birth [28]. In adult lungs, AT1 cells marked by the transcription factor HOPX have the potential to act as a facultative progenitor capable of becoming SP-C$^+$ AT2 cells and contribute to compensatory lung regrowth after pneumonectomy in mice [29]. As new experimental evidence continues to provide new insight into how the lung maintains and repairs itself, the established paradigms must also shift.

The continued evolution of traditional cell hierarchy and potential for repair will be important in developing new cell-based therapies for lung regeneration. The ability to reactivate and exploit these complex endogenous cell repair mechanisms for therapeutic benefit continues to be a major goal of regenerative medicine (Fig. 60.2).

NOVEL CELL POPULATIONS FOR LUNG REPAIR

As an internal organ that allows for direct and noninvasive access to the respiratory tissue, delivery of regenerative cells as therapy for airway disease is an attractive concept. However, innate epithelial defense and clearance mechanisms mean that delivered cell engraftment is generally low and transient in nature. Robust methods are required to track and quantify any surviving cells, which often includes luciferase or fluorescent transgene expression coupled with ex vivo tissue analysis by quantitative polymerase chain reaction or histology [30]. These methods are prone to error owing to sampling methods and the potential for identifying false-positives, which makes the conclusions difficult to interpret. The field continues to advance toward the goal of directed cell therapy to the airways as new methods are developed and new cell populations are identified. Intravenous infusion is an alternate route to achieving cell delivery of regenerative cell populations to the lung. The pulmonary capillaries have a mean diameter of 5.5 μm, which means that as larger cells pass through the vascular network, a high percentage can be retained

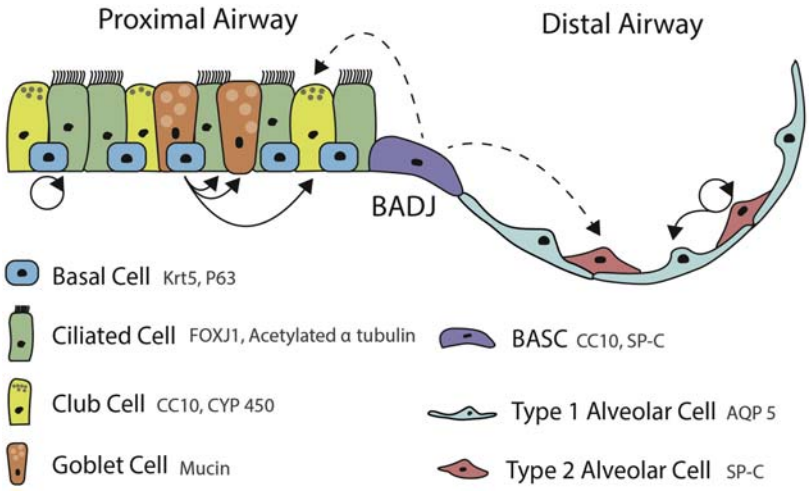

FIGURE 60.2 **Endogenous lung epithelial repair.** Schematic of the lung epithelium from a proximal to distal location. The location and function of important endogenous lung epithelial stem/progenitor cell populations are listed, which can be activated to differentiate and self-renew to maintain tissue homeostasis. *Arrows* indicate well-established (*solid*) and novel (*dashed*) differentiation pathways. *AQP 5*, aquaporin 5; *BADJ*, bronchoalveolar duct junction; *BASC*, bronchoalveolar stem cells; *CYP 450*, cytochrome 450; *SP*, surfactant protein.

within the lung and can exert local effects [31]. In addition, many reports have suggested that exogenous cells arising from the bone marrow can be mobilized into the peripheral blood and may engraft to repair injured lung tissue. This paradigm remains controversial, and robust conclusions from these studies also require rigorous experimental analysis. Cell lineage-tracing experiments have suggested that the long-term contribution of bone marrow−derived cells to the lung epithelium [32] or endothelium [33] is low or undetectable.

A separate population, the mesenchymal stem/stromal cells (MSCs), have been widely studied as a reparative population for lung cell therapy. This nonhematopoietic multipotent stromal population can be isolated from various biological sources, including bone marrow, peripheral blood, adipose tissue, dental pulp, and umbilical cord blood, and are initially defined by plastic adherence in vitro [34]. These cells, marked by CD105, CD73, and CD90 expression, also secrete antiinflammatory cytokines, including interleukin (IL)-10 and can decrease proinflammatory cytokine expression, such as IL-1β, IL-6, and interferon-gamma [35]. When delivered intravenously, MSCs can act in a targeted way onto the lung after injury. Treatment with MSCs has shown benefit in animal models of fibrosis after bleomycin induced lung injury, significantly reducing inflammation and collagen deposition within lung tissue [36,37]. Use of these cells is also being translated to clinical application. A proof-of-principle trial was completed that demonstrated the feasibility of treating pulmonary fibrosis by intravenous infusion with MSCs [38], and many clinical trials are ongoing. MSC may also have the potential to treat sepsis and acute respiratory distress syndrome in patients [39] and have shown benefit in repairing donor lungs deemed otherwise unsuitable for transplantation [40]. Taken together, this multifaceted ability to regulate the immune response and enhance tissue repair after lung injury makes MSC-based therapy a promising and advancing area of regenerative medicine.

Epithelial injury is an important driver for regenerative cell activation and is a critical component of many experimental models. Tissue damage may in fact be essential for regeneration, which suggests that preconditioning injury of the lungs may be required before cell therapy. One study of endogenous lung repair during the canalicular stage of development used sublethal radiation injury to vacate the putative lung progenitor niche [41]. It was reported that intravenous infusion of lung-derived cell populations resulted in long-term lung chimerism, which was identified in clonal donor-derived patches. Treatment with these cells after injury resulted in a marked improvement in lung function compared with untreated controls. In addition, injury has been shown to activate specific facultative progenitor populations, which can also be used for therapeutic treatment. A study of lung repair after H1N1 influenza-induced lung injury showed that lineage-traced keratin (Krt)5^+ cells undergo a proliferative expansion to regenerate the damaged tissue. After isolation of these cells and subsequent intratracheal delivery 5 days after influenza infection, tissue analysis revealed that multiple epithelial lineages in the regenerating lung were derived from the transplanted cells. These included both AT1 and AT2 pneumocytes at 40 and 90 days after influenza infection [42], which suggested that traditionally proximally specified $Krt5^+$ cells take on a bilineage potential after injury. Parallel work identified a rare lineage-negative $Krt5^+$ cell population in normal distal lung tissue that is also activated after influenza- or bleomycin-induced lung injury and could also migrate to repair the injured tissue [43]. After orthotopic

transplantation of these cells to influenza-injured lungs, multicellular structures were identified in areas of active regeneration, which also observed differentiation toward SP-C$^+$ distal epithelial fate. These studies further revealed that the Notch signaling pathway has a critical role in proximal versus distal epithelial fate decisions during regeneration and tissue repair. These new insights into epithelial cell potential and regenerative capacity further refine the optimal cells for therapeutic use.

The directed differentiation of pluripotent stem cells toward defined lung lineages is another exciting option for generating cells suitable for use in lung tissue engineering. These cells also offer a valuable opportunity to model lung development and respiratory disease using human cells. Much research and progress have been accomplished using both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) to recapitulate the developmental program and drive specification of lung-specific cells. Early studies first attempted to a drive a lung epithelial phenotype from ESCs by culture in small airway growth media [44] and transition to an air−liquid interface (ALI) culture [45]. Coculture with digested fetal lung mesenchyme was able to induce differentiation of a distal SP-C$^+$ epithelial population from murine ESCs, which also highlights the critical role of growth factor cross-signaling during development [46]. Increased lung differentiation efficiency can be accomplished by first stimulating the Nodal pathway with activin A treatment, to induce a definitive endodermal germ layer fate directly [47,48]. Additional patterning of the endoderm toward the anterior foregut by inhibiting the transforming growth factor-β and bone morphogenic protein signaling pathways further aids specification of a lung-specific fate [49]. Two important and parallel reports described the successful differentiation of NK2 homeobox 1 transcription factor (Nkx2.1)-expressing lung/thyroid progenitor cells from this anteriorized foregut population [50,51]. Further differentiation of a lung-specified population can generate varying degrees of mature proximal (CC10$^+$ and p63$^+$) and distal (SP-C$^+$) epithelial lineages in vitro. Ideally, for use in cell therapy or tissue engineering applications, the differentiation methods would be able to generate a large number of homogeneous cell populations. The continued optimization of targeted and robust protocols to generate specific epithelial cell lineages is a challenge in this rapidly advancing field.

An important study reported that after a differentiation period of 48 days, human ESCs were able to produce basal, goblet, Clara, ciliated, and both type I and type II alveolar epithelial cells. When the progenitor cell potential was tested in vivo by implantation under the kidney capsule in mice, cystic and tubular structures were found lined by an epithelial layer of both pseudostratified and elongated morphology [52]. Human iPSCs can also be differentiated to create a functional multiciliated proximal epithelium after 28 days of culture at ALI [53]. In this study, expression of the cystic fibrosis transmembrane receptor (CFTR) could be visualized at the apical surface of polarized cells. These results demonstrate the capacity of defined stem cell populations to create functional tissue and the exciting potential these cells can have in regenerative medicine for lung diseases. Starting with a patient-specific pluripotent cell affords the possibility of correcting or enhancing these populations before differentiation. This is particularly attractive for pulmonary diseases resulting from monogenic mutations, such as cystic fibrosis (CF). It has been shown that starting from a small CF patient skin biopsy, iPSCs can be generated and corrected for the ΔF508 mutation in the endogenous CFTR gene, using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-associated protein 9 system. Subsequent differentiation of these iPSCs to a proximal airway epithelial fate, including culture at ALI, further showed the ability to restore the CFTR chloride current by patch clamp studies [54]. The potential for CFTR gene correction using zinc-finger nucleases combined with homology-directed repair has also been successfully demonstrated [55]. These exciting advances in cell and molecular biology have great promise in patient-specific regenerative medicine. The combination of these novel technologies with parallel advances in cell therapy and tissue engineering has the potential to have a meaningful impact for patients in need.

BIOLOGICAL SCAFFOLDS TO SUPPORT REGENERATION

As knowledge of regenerative cell populations advances, the challenge of engineering a functional three-dimensional (3D) tissue still faces many hurdles. The use of biological scaffolds can further support these cells in creating lung tissue ex vivo by providing a platform to enhance regeneration in an anatomically correct environment. The choice of biomaterial and the methods used to create these lung scaffolds are important considerations that are integral to the ultimate function of the regenerated tissue.

Artificial scaffolds have been used for lung and airway tissue engineering. In one study, fetal rat lung cells were combined with Gelfoam and injected into normal healthy lung parenchyma; they showed cell survival for 35 days and provided a supportive environment for recipient-derived cells to engraft [56]. Scaffolds can also be used to sequester cell-derived growth factors for targeted delivery. This was demonstrated with adipose-derived MSCs

grown on a polyglycolic acid felt sheet, which secreted large amounts of hepatocyte growth factors and resulted in enhanced compensatory lung growth after surgical resection. Also of note are the efforts to create a synthetic, implantable tracheal graft. The relatively simple tissue, which is composed of a hollow tube with compatible mechanical properties, seems a promising first goal toward patient-specific regenerative medicine. The minimal requirements for any biomaterial used for this purpose would be biocompatibility to the recipient and suitability as a substrate for cell attachment and survival [57]. A large number of biocompatible materials have been tested for this purpose, including biodegradable polymers (polyglycolic acid, polylactic acid, and polycaprolactone), and hydrogels (Pluronic F-127 and collagen gel). Important progress has been made using the nanocomposite biomaterial polyhedral oligomeric silsesquioxane poly(carbonate-urea) urethane. This material has been shown to be biostable, to have favorable mechanical properties, and to support the growth of various cell types, including human bronchial epithelial cells [58]. In addition to synthetic materials, decellularized native tracheas are promising scaffolds for airway engineering. Decellularized tissue engineered tracheas have been investigated in a number of studies using various species, with mixed results [59]. Although good epithelialization was observed in many reports, grafts were structurally unstable, leading to airway obstruction and subsequent collapse. An important consideration for large airway engineering is the long-term maintenance of biomechanical integrity and lumen patency, to prevent the need for subsequent dilatation or stenting of the implanted graft.

When aiming to create more complex tissues such as functional lung parenchyma, the ability to create a suitable scaffold with adequate tissue architecture and resolution presents significant challenges. The concept of whole-organ decellularization provides a promising option. Removal of all the cells from a tissue, leaving the only the extracellular matrix (ECM), is a long-standing technology, and acellular ECM scaffolds are used in clinical practice for many applications [60]. Many of these are products derived from the dermis, pericardium, small intestinal submucosa, and urinary bladder of bovine and porcine sources. The ability of these ECM scaffold to facilitate constructive tissue remodeling in vivo makes them a valuable tool for regenerative medicine.

Building on this concept and stemming from a seminal article on whole-heart regeneration [61], this approach was first applied to whole lungs in an exciting series of reports in 2010 [62–64]. By perfusing a detergent through the native vasculature of the lungs, ideally at a controlled pressure, the intact organ can be fully decellularized, leaving only the ECM scaffold. Detergents used include sodium dodecyl sulfate, sodium deoxycholate, and 3-([3-cholamidopropyl] dimethylammonio)-1-propanesulfonate. Comparative analyses of these detergents for use in decellularization have not provided a consensus and suggest that many approaches are valid [65–67]. Lung decellularization has been upscaled and applied across many species including mice [68], rats [69], pigs [66], nonhuman primates [70], and humans [66,71]. It has been confirmed that the acellular scaffolds retain essential collagens, laminin, elastin, and glycosaminoglycan to support cell reattachment [66,72]. The relevant importance of the ECM composition versus structure is an area of active study and interest. Proteomic mass spectrometry has been applied to analyze and compare the protein scaffold that is retained after decellularization [66,72,73]. These studies provide important insight into the composition of the bioactive scaffold upon which new tissue will be created. If essential proteins are lost through the decellularization process, or if immunogenic proteins are retained within the matrix, there can be significant consequences for the regeneration process both during ex vivo recellularization and after in vivo implantation [74]. A quantitative comparison of the acellular scaffold with the ECM of native lung tissue was achieved by applying a stable isotope-labeled concatemer approach, in which known concentrations of labeled peptides were incorporated to generate a standard reference curve [75]. Further refinement of these techniques, as applied to diverse biological scaffolds, will provide more precise information about the relationship between protein composition and overall tissue regeneration and function.

The source of native lung tissue used to prepare scaffolds can have a direct impact on subsequent regeneration. Several studies have shown that underlying lung pathologies can cause changes in the ECM that are retained after decellularization and can perpetuate during tissue repair [76]. Remodeling of the ECM is a hallmark of pulmonary fibrosis characterized by increased collagen deposition and changes in the alveolar architecture. When normal human lung fibroblasts were grown on ECM scaffolds derived from fibrotic lung tissue, genes associated with fibrotic changes became activated [77]. This suggests that the origin of the ECM can have a great impact on cellular function and fate. It has also been shown that culture on fibrotic ECM can drive myofibroblast differentiation from normal lung fibroblasts [78]. Emphysema also dramatically affects the lung matrix composition and structure. In a murine model of emphysematous lung injury induced by treatment with porcine pancreatic elastase, the subsequently decellularized lungs portrayed a unique pattern of ECM retention compared with uninjured lungs. Despite these histologic and compositional differences, no significant differences were noted when MSCs or $CC10^{+}$ epithelial cells were redelivered to the emphysematous lung scaffolds [79]. Similar changes in scaffold architecture were reported in human emphysematous lung scaffolds; in these studies, subsequent cell attachment and survival were significantly

decreased in the diseased scaffolds compared with those from normal lungs [80]. Interestingly, when the acellular lung ECM was solubilized and prepared as a coating to support 2D cell grown in vitro, no differences in survival were found, which suggests that the 3D architecture of the acellular lung scaffold is the main effector of these changes. The age of the lung can contribute important differences to the decellularized scaffold. In one study of aged versus young mouse lung scaffolds, cells grown on the older ECM were found to express a significantly lower level of laminins α3 and α4, which recapitulates the laminin deficiency that is observed in aged lung ECM. These data further highlight the deep biological information that is contained with lung scaffold and the feedback loops that can exist between reparative cell populations and the underlying ECM [81].

There has not been a systematic analysis of these ECM-associated variables, and those that were studied varied across methodology, species, and the cell type used. This level of investigation will be necessary before clinical translation of decellularized lung scaffold-based tissue engineering, to standardize and optimize the biological source of lung ECM and exclude factors that may be detrimental to regeneration.

One possible way to reduce variation in the lung ECM scaffold would be to use animal sources of donor organs. The ability to control age, size, and any variation in environmental exposure or potential pathology would standardize the starting material for clinical lung tissue regeneration. One widely explored option is the porcine lung, because pig organs are anatomically similar to human organs. Yet, there are also many important differences between the species. One study comparing decellularized scaffolds from rat, pig, primate, and human lungs found that human and primate lungs were stiffer, contained more elastin, and retained fewer glycosaminoglycans than did pig or rat lung scaffolds [82]. Adhesion of human endothelial cells in the vascular network of the lung scaffold was also markedly enhanced in human and primate tissues compared with porcine and rat matrices. These finding reaffirm that the ECM composition can have an important role in tissue regeneration after recellularization. There are also anatomic and morphologic differences between human and porcine lungs [83]. Of note, porcine lungs lack interalveolar connections known as pores of Kohn, which facilitate collateral ventilation and prevent atelectasis. When choosing sources of lungs for xenotransplantation, it may be important to consider whether the animals are quadruped or bipedal, because the mode of ambulation can also affect breathing mechanics.

The use of porcine lung xenografts for transplantation is also complicated by hyperacute rejection, mediated by antibodies anti-Gal (Galα1-3Galβ1-(3)4GlcNAc-R), an epitope that humans do not express. Exciting research has suggested that this rejection may be overcome with the use of genetically modified Gal knockout animals [84]. Proof-of-concept studies demonstrated that decellularized wild-type and α-Gal knockout pig lungs can be comparably decellularized and are biocompatible with human lung cells despite some differences in retained proteins [85].

Another intriguing source of scaffolds for lung regeneration is 3D printing, also termed stereolithography, to create complex solid organ matrices [86]. This approach would allow for custom-designed organs to be created for the intended recipient, further augmenting the concept of personalized medicine. Through bioprinting, the biomaterial can also be combined with cells in a controlled and localized manner, which provides a potential advantage over the random repopulation of a decellularized scaffold. Major challenges facing adoption of this technology for whole-lung regenerative medicine are resolution, scale, and the composition of the construct.

ADVANCES IN REBUILDING FUNCTIONAL LUNG TISSUE

Higher-level tissue function is a critical step in regenerating tissue for clinical application. To engineer complex tissue ex vivo, the cells used must retain their regenerative capacity for repair and differentiation when recombined. Several in vitro methods have been developed to study and validate the regenerative capacity of important cell populations. This ability is a function of not only of the cells themselves, but also of the microenvironment in which they are placed. The ability for cells of the respiratory tract to form tight junctions is critical for maintaining fluid balance and controlling solute transport. In vitro, barrier function is typically measured by growing cells on semipermeable inserts and quantifying transepithelial electrical resistance (TEER) [87]. The application of an ALI culture environment further drives tissue maturation and cellular differentiation toward a ciliated phenotype, which can be used to further enhance tissue regeneration.

A 3D culture system can also be used to test the regenerative capacity of specific lung cell populations in vitro. In one model, isolated and purified basal epithelial cells demonstrated the ability to formation of clonal "tracheospheres" containing both ciliated and secretory cells [17]. Single, isolated murine AT2 cells can also create 3D alveolar-like structures termed "alveolospheres" [27]. In this model, the presence of primary platelet-derived growth factor receptor α-positive mesenchymal support cells was essential to recapitulate the regenerative niche and support 3D tissue formation. Airway organoids have also been created by combining human adult primary bronchial

epithelial cells, lung fibroblasts, and lung microvascular endothelial cells, which demonstrated self-organization into discrete structures and recapitulated limited aspects of branching morphogenesis [88]. These methods provide a useful experimental tool to study the ex vivo potential of specific cell types and assess their ability for complex tissue regeneration. In this context, the surrounding microenvironment and exogenous factors can easily be tested and manipulated to better define and enhance the stimuli needed to drive tissue regeneration. Such 3D models can also be used to study lung development. Murine E17.5 lung cells can be isolated and cultured in 3D collagen hydrogels to create distal proSP-C–expressing epithelial structures surrounded by a capillary-like endothelial network [89]. This system can be used to test the influence of critical growth factors and ECM components on lung morphogenesis. The establishment of multilineage lung organoids from iPSC-derived progenitors has also been reported, by directing differentiation along the defined steps of lung development [90]. The successful engraftment, long-term survival, and maturation of organoid-derived lung epithelium in vivo is also important for therapeutic applicability. The use of synthetic scaffolds to support survival and regeneration may confer an important advantage to cells after implantation [91].

Small matrix slices can also be used to study tissue regeneration in a high-throughput manner. After decellularization, small pieces of lung tissue can be sectioned (250 μm) and placed into traditional 2D culture. Recellularization of these thin lung matrix slices demonstrated biocompatibility and integrin-mediated cell attachment to the ECM [66]. The effect of ECM composition on tissue regeneration can also be investigated using the lung matrix slice model. In one study, when ESC-derived definitive anterior endoderm cells were cultured on ECM that was pretreated with Heparitinase I to remove matrix-associated heparan sulfate proteoglycans, cellular organization and differentiation were compromised [92]. This suggests that retention of important ECM components after decellularization may be required to support mature tissue regeneration and function. Native 3D lung ECM was further shown to promote lung lineage specification from human iPSCs, as identified by Nkx2.1 expression, compared with traditional 2D in vitro differentiation [93]. Cells can also be delivered to whole rodent lung scaffolds in a mixture of agarose, creating a semisolid tissue that can be further sectioned and maintained in vitro, as described using lung-specified murine ESCs [50]. A method of excising small segments ($\sim$1–3 cm^3) of human decellularized lung tissue that retains 3D vascular and airway conduits has been described and used for recellularization in a high-throughput in vitro model [72]. The clear benefits of these smaller-scale models to test cell differentiation and tissue regeneration is the ability to test more conditions with less variation while also reducing experimental time and costs.

The native lung scaffold is particularly well-suited for recellularization because it retains the anatomical separation between the airways and vasculature network while enabling direct access to both compartments. Epithelial or mesenchymal cell populations can be delivered to the respiratory tract via the trachea, and endothelial cells can be delivered via the pulmonary artery and vein, ultimately repopulating all of the essential cell types in the lung without compromising the underlying organ structure. The first studies to prove biocompatibility of the whole decellularized scaffold used a heterogeneous mixture of pulmonary epithelial cells isolated from neonatal rats [63,64]. This important proof-of-principle has evolved to include studies using a wide range of primary and pluripotent-derived cell populations. This methodology has been successfully applied to regenerate human lung scaffolds repopulated with donor tissue-derived basal epithelial stem cells and pulmonary artery endothelial cells. These recellularized tissues were maintained in ex vivo culture for over 7 days, confirming cell viability and continues proliferation while reforming the complex lung tissue architecture along the native matrix [94]. A challenge remains to derive the optimal cells for use in regenerating these scaffolds. Primary epithelial cells are a convenient possibility because they can be isolated from small tissue biopsies or airway brushing, but expansion to an adequate cell number in vitro can be limiting. The modification of important cell pathways, including the Rho-associated protein kinase and SMAD, can be achieved by adding targeted small molecules inhibitors to the culture media to reduce cell senescence and maintain proliferation [95,96]. An additional challenge inherent in patient-derived cells is the contribution of underlying pathologies, which may compromise cell isolation, expansion, or function.

Combined with advances in cell and matrix biology, the feat of bioengineering fully functional lungs and airways for transplantation will require the continued optimization of many additional factors. Implementing these advances to recreate higher-level tissue function will also depend on developing successful methods to culture and mature the organ ex vivo before implantation. When creating systems for lung culture outside the body, much can be learned from the ex vivo lung perfusion technology, which is used in clinical lung transplantation to expand the donor pool [97]. This procedure maintains cadaveric donor lungs with physiologic protective ventilation and perfusion, with the aim of facilitating more accurate assessment of the graft. This system, which is typically applied for 3–4 h before transplant, can also serve as a platform to recondition lungs that would otherwise be deemed unsuitable for transplantation, or to apply novel treatments such as gene therapy with the immunosuppressive

cytokine IL-10 [98]. These clinically relevant advances in ex vivo lung preservation have provided important benefits to patients but are still limited by a relatively short time in which the lung remains stably functional.

Applying some of these concepts to ex vivo lung engineering has guided the development of lung bioreactor systems to support the recellularization and culture of biological scaffolds with the aim of establishing and maintaining lung function on the scale of days to weeks. Effectively recapitulating the biomimetic events associated with breathing and blood perfusion in an ex vivo environment is a critical component of successful lung bioengineering. The optimal choice of perfusate is an important consideration that has not yet been robustly evaluated or defined. Not only must the perfusate facilitate nutrient delivery and waste removal through the regenerating vessels, it must prevent unwanted vascular permeability and tissue edema, for which traditional cell culture medias are not optimal. Methods for lung vascular regeneration have been described using both primary and iPSC-derived endothelial cells, which require specific alterations to the perfusion media composition to induce angiogenesis or vascular barrier function during defined phases of regeneration [99]. Further development and improvement in vascular regeneration are important and complementary component of respiratory tract tissue engineering. It has been shown that during whole rodent lung recellularization, the coculture of human lung-derived endothelial cells with primary basal cells imparts a proliferative advantage to the regenerating epithelium [94]. An additional challenge of using traditional cell culture media for complex tissue engineering is defining a composition that is optimized to support multiple cell types (Fig. 60.3).

Many versions of ex vivo lung bioreactors have been developed and tested, ranging from the rodent to the human scale [100–102]. The minimal requirements are to provide a controlled environment with proper temperature and humidity, and to maintain sterility. The application of specific biomechanical stimuli during organ culture in the bioreactor can have direct effects on the tissue and cells, and so careful control of both perfusion and ventilation are important design parameters. Sheer stress applied to the regenerating vasculature by perfusion can act to align and stimulate pulmonary endothelial cells after redelivery to the scaffold [103]. Perfusion must also provide

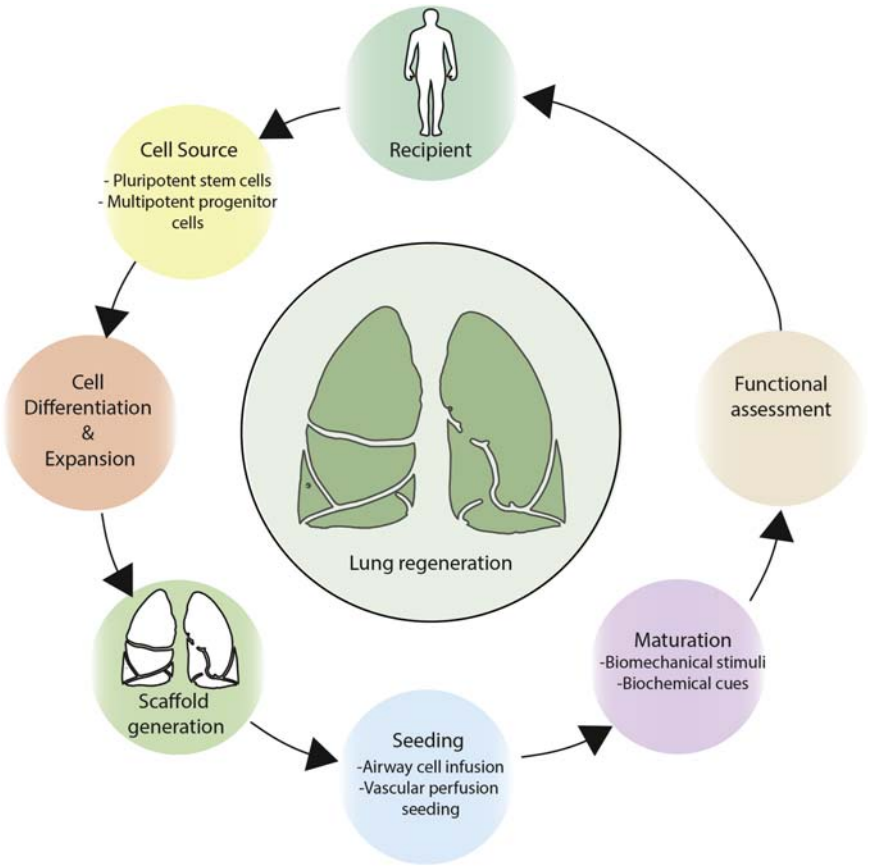

FIGURE 60.3 **A road map for lung regeneration.** Essential steps and current challenges in personalized regenerative medicine for the lung are listed, with the aim of bioengineering functional lung tissue for therapeutic use.

adequate delivery of oxygen and nutrients to the regenerating tissue. The cyclic stretch and relaxation that is created during mechanical ventilation can induce biological changes in lung epithelial cells, including surfactant secretion, differentiation, and cytokine release [104]. As described in vitro, the inclusion of an ALI between the perfused vasculature and the inflated airways during whole-organ culture can also be used to induce ciliation of the proximal airway epithelium. The protocols dictating when to apply these biomimetic stimuli and at what magnitude are not yet defined. Establishment of robust methods will also require sufficient analysis techniques to assess for cell viability and tissue regeneration in a noninvasive manner during culture. One reported method uses a resazurin-based reduction assay delivered through the vascular perfusate. Fluorescence of resazurin-containing medium can be used as a correlate of cellular metabolism, which is consistent with histological assessment of the regenerating tissue [105]. Measurement of glucose consumption and lactate production in the media during organ culture can also be used as noninvasive indicator of cell viability. Biological tests such as these, combined with a functional analysis of graft gas exchange capacity and perfusability, can be used to establish meaningful, quantitative criteria for transplant readiness.

CLINICAL TRANSLATION AND FUTURE CONSIDERATIONS

Lung transplantation remains the only curative treatment for advanced lung diseases; yet, insufficiencies in donor lung availability and long-term challenges with graft rejections continue to limit the therapeutic utility. Although advances in organ preservation, reconditioning, and immune tolerance provide important benefits, the ability to harness regenerative medicine to create lung tissue for clinical use is an exciting and possibly complementary option. To this end, many challenges have been discussed in this chapter. This includes harnessing endogenous cell repair mechanisms, improving methods to derive pulmonary cells from pluripotent progenitors, and developing methods to create new tissue constructs based on clinical-scale biomaterials, in addition to validating protocols for maturing and evaluating the regenerated tissue. The role of in vivo factors that drive repair has been mostly described in the context of animal lung injury models. The contribution of these mechanisms to tissue repair after implantation of an engineered tissue is widely unstudied in the lung and will likely have an important role in regenerating the respiratory tract.

The ability to direct regeneration, either ex vivo or in vivo, from patient-derived cells, would have significant benefit to people with lung disease. The capacity to apply these regenerative medicine approaches in an on-demand fashion is an additional advantage of these novel technologies. Much work is still required to coalesce the fields of lung development, cell biology, and tissue engineering fully, but promising advances have already been realized. Important questions regarding cell potential and the contribution of the microenvironment are being studied and applied to advance the goal of functional regenerative medicine for the lung.

List of Acronyms and Abbreviations

2D/3D two or three dimensions
ALI Air–liquid interface
AT 1/2 Alveolar type 1/2 pneumocyte
CC10 Club cell secretory protein, 10 kD
CF Cystic fibrosis
CFTR Cystic fibrosis transmembrane receptor
CRISPR Clustered Regularly Interspaced Short Palindromic Repeats
ECM Extracellular matrix
ESC Embryonic stem cell
FGF Fibroblast growth factor
H1N1 Influenza A virus
IL Interleukin
iPSC Induced pluripotent stem cell
Krt Keratin
MSC Mesenchymal stem or stromal cell
NEB Neuroendocrine body
Nkx2.1 NK2 homeobox 1 transcription factor
p63 Transformation-related protein 63
SP Surfactant protein

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CHAPTER

61

Cardiac Tissue

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INTRODUCTION: FROM TISSUES TO ORGANS: KEY GOALS AND ISSUES

Nearly 8 million people in the United States have experienced a myocardial infarction (MI). Each year, 665,000 new cases occur [1]. MI results in the substantial death of cardiomyocytes in the infarct zone, followed by pathological remodeling of the heart. The remodeling process involves cardiac dilation, wall thinning, and severe deterioration of contractile function, leading to congestive heart failure in more than 900,000 patients each year in the United States [1]. Conventional therapies are limited by the substantial inability of myocardium to regenerate after injury [2] and the shortage of organs available for transplantation. This chapter will focus on describing approaches that have been considered as novel treatment options after MI [3].

Regardless of the approach to regenerative medicine or the scope of the application (a vascular graft, a pediatric valve, or an entire heart), there are three overlapping therapeutic goals: the three R's:

- Increase the safety of tissue and organ **replacement** and its effectiveness, and make it more widely available;
- **Repair** tissues and organs without having to replace them; and
- Enable tissues and organs to **regenerate** so that repair and regeneration become one and the same.

Furthermore, the problems of reaching these goals can be summarized (Table 61.1) in three categories (here largely in the context of tissue engineering) [4,5]:

- **Cell number**: What is the source of cells to be used and how will large numbers be generated? How will they be supplied with nutrients and oxygen (and have wastes removed) within a device of reasonable volume?
- **Cell function**: How will the scaffold, extracellular matrix (ECM), and diffusible factors interact to generate the desired cell phenotype? How will the engineered tissue or organ integrate with the host to ensure a functional outcome?
- **Cell durability**: What will happen over the long term as remodeling and/or the host immune or inflammatory system responds to the new tissue?

To replace, repair, or regenerate cardiovascular tissue, these central issues of regenerative medicine will need to be addressed. Some of these issues (Table 61.1) reflect the fundamental nature of how an organ is different from a tissue: the large size and three-dimensional (3D) structure, and the presence of multiple cell types that work in unison. A number of cell sources are available for cardiac tissue engineering, ranging from primary cardiac cells to stem cell differentiated cardiac cells. Advantages and disadvantages of cell sources are listed in Table 61.2. Beyond these largely scientific challenges, there are the no less critical, practical questions of manufacturing, sterilization, storage, and distribution, and the regulatory and public policy issues that will need to be addressed before such therapies can be made available to the patients who are expected to benefit. Furthermore, we will need new imaging or other noninvasive strategies to monitor the success (or not) of these therapies: i.e., to enable the translation into clinical practice.

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TABLE 61.1 Critical Issues Associated With Tissue Engineering a Heart [5]

	Objective	Critical Issues
Cell number	• ~300 g cells (3×10^{11} cells) • ~200 mL O_2/h[a]	• Cell source/purity • Vascularization
Function	• Cellular phenotype (multiple cell types) • Coordinated muscle contraction • Pump blood • Connect to circulation	• Microenvironment (soluble and insoluble factors) • Pacemaker and electrical conduction • Valves and conduits • Biomechanical elasticity and strength • Nonthrombogenicity
Durability	• Fatigue resistance • Hypoxia and disease tolerance • Host tolerance	• Biocompatibility • Remodeling • Innate/adaptive immune response
■ Manufacturing and quality control		
■ Ethical, legal, and social issues		
■ Imaging and noninvasive diagnostics		
■ Regulatory and public policy issues		

[a]*Based on moderate activity [5a].*

TABLE 61.2 Cell Sources for Cardiac Tissue Engineering and Some of Their Advantages and Disadvantages [5]

Cell Sources	Advantages	Disadvantages
Adult cardiac cells	Target cell source	Little proliferative or developmental potential; limited resources
Fetal cardiac cells	Some proliferative potential, appropriate developmental potential; demonstrated efficacy	Limited resources; ethical considerations
Endothelial progenitor cells	Some proliferative potential; may elicit in vivo healing through indirect mechanisms	Appropriate developmental potential yet to be demonstrated; may not be appropriate for larger tissue replacement or in vitro tissue engineering
Adult bone marrow–derived cells	Significant in vitro proliferative potential; some demonstration of efficacy	Appropriate developmental potential to be demonstrated; safety tolerance after in vitro culture to be determined
Embryonic stem cells Induced pluripotent stem cells	Significant in vitro proliferative potential; demonstration of efficacy; appropriate developmental potential; sustainable resource	In vitro culture may introduce genetic changes; safety tolerance after in vitro culture and differentiation to be determined

ENGINEERING OF CARDIAC PATCHES USING CELLS, SCAFFOLDS, AND BIOREACTORS

Whereas small infarcts may be treated with cell therapies, larger areas of damaged tissue may require replacement with a cardiac patch. The time after an infarction is critical in the success of any regeneration strategy. Upon MI, a vigorous inflammatory response is elicited and dead cells are removed by marrow-derived macrophages. Over the subsequent weeks to months, fibroblasts (FBs) and endothelial cells (ECs) proliferate, forming granulation tissue and eventually a dense collagenous scar. Formation of scar tissue severely reduces the contractile function of the myocardium and leads to ventricle wall thinning and dilatation, remodeling, and ultimately heart failure. Thus, the best regeneration strategy depends on the length of time passed since the incidence of the infarction; i.e., new and old infarcts most likely cannot be treated using the same approach.

Cell injection strategies, will work best if applied shortly after MI. Application of cells and growth factors within hours and days after the MI has the potential to direct the wound repair process so that the minimum amount of scar tissue is formed, the contractile function is maintained in the border zone, and pathological remodeling is

attenuated. Tissue engineering strategies will work in the acute phase as well, but may be more necessary after a scar has formed. In these cases, larger areas of the heart must be replaced or augmented, and this is potentially where a scaffold-based approach may be most useful.

Scaffold-based therapy can be divided into two approaches: (1) a hydrogel, in which cells are either encapsulated and cultivated in vitro or injected directly into the MI area without preculture; or (2) porous and fibrous 3D scaffold, in which scaffolds are seeded with cells and in most cases cultivated in vitro before use as cardiac patches. Natural ECM may also serve as a scaffold. In addition, repair of the heart with biomaterials alone or constructs made by cell self-assembly has also been examined and performed.

Patients with large transmural akinetic scars often benefit from the Dor procedure (endoventricular circular patch plasty) [6,7]. In some cases, however, the success of this procedure is temporary, thus motivating the need for viable tissue patches. In this procedure, the scar tissue is excised and the ventricle is closed using a circular Dacron (polyethylene terephthalate) patch lined with endocardium.

In a pioneer study, Taylor and colleagues used the ECM of the native rat heart as a scaffold for cardiac tissue engineering [8]. This approach enabled them to preserve the underlying geometry and create an ideal natural template for tissue engineering of the heart (Fig. 61.1). The authors decellularized adult (12-week-old) cadaveric Fisher rat hearts by coronary perfusion with detergents (Fig. 61.1A). In addition to conserving the ECM, the vasculature was preserved and was perfusable (Fig. 61.1B). The structure of the ventricles, atria, and heart valves was also preserved. Cardiomyocytes were then isolated from neonatal rats and reseeded onto the structure. Vascular perfusion with the oxygenated media was provided via a peristaltic pump. In a subgroup of samples, rat aortic ECs were injected into the aorta to recellularize the vasculature. Macroscopic contractions were observed by day 4 of cultivation, whereas a pump function of about 2.4 mmHg was generated at day 8 under electrical

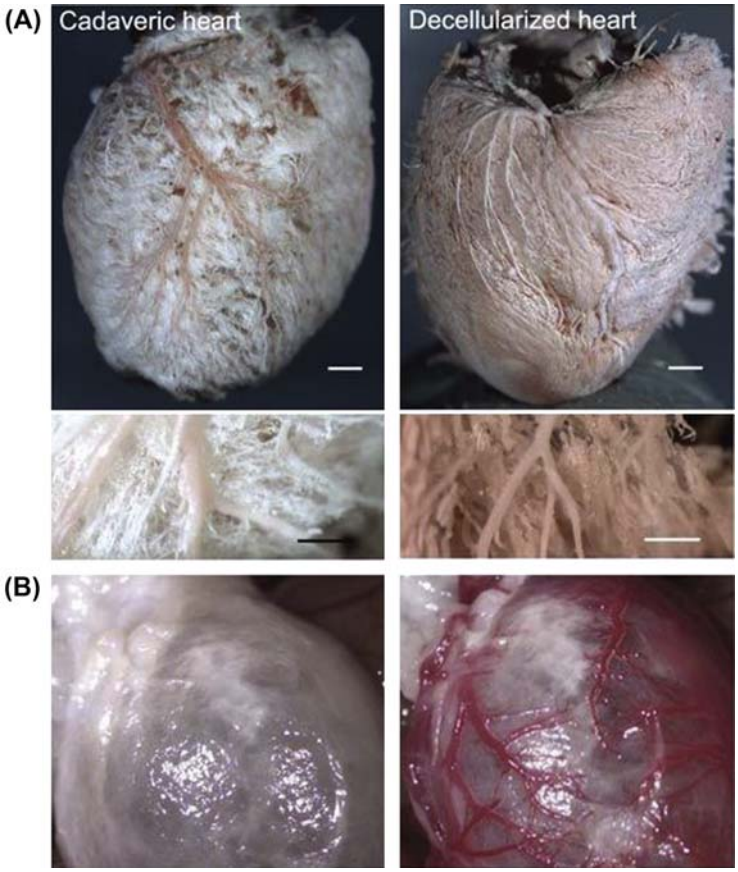

FIGURE 61.1 **Native heart extracellular matrix can serve as a suitable substrate for tissue engineering of a whole heart.** (A) Low-magnification (upper row; scale bar = 1000 mm) and high-magnification views (lower row; scale bar = 250 mm) of coronary corrosion casts of cadaveric and decellularized whole adult rat hearts. (B) Upon heterotropic transplantation of the decellularized whole rat heart before (left), blood can be seen flowing through the preserved decellularized vascular structures shortly after unclamping of the host aorta (right) [8].

stimulation. Clearly, the performance was only a small fraction (~2%) of the native heart; nonetheless, this was a milestone in cardiac tissue development.

In a similar approach, Guyette et al. decellularized human cadaveric hearts, leaving behind the ECM components [9]. The decellularized heart had with mechanical properties similar to those of the cadaver tissue. The remaining human cardiac matrix was perfused with induced pluripotent stem cell (iPSC)-derived cardiomyocytes. After 14 days of culture, although it was immature, the repopulated human heart was metabolically active and responsive to electrical stimulation. Further work involving full recellularization of the myocardial matrix and valves, as well as long-term organ culture to assist in tissue maturation is required before this technique can be brought to the clinic; however, it serves as foundational work in the path to full organ regeneration and repair.

In cardiac tissue engineering approaches, most studies suggest that some type of scaffold, an inductive 3D matrix, is necessary to support the assembly of cardiac tissue in vitro. An important scaffold-free approach includes stacking confluent monolayers of cardiomyocytes [10]. Although cardiac patches obtained in this way generate a high active force, engineering patches more than two to three cell layers thick remains a problem. Shimizu and colleagues described a polysurgery approach in which vascularized cardiac grafts can be created by sequentially layering cell sheets in multiple surgeries spaced at 1- to 3-day intervals [11]. Although this approach demonstrates that thick tissues (~1 cm) can in principle be created from cell sheets, the approach will be difficult to implement in the clinical setting. Murry and colleagues managed to obtain a cardiac patch based on human embryonic stem cell (ESC)-derived cardiomyocytes by the self-assembly of isolated cells in orbitally mixed dishes [12], essentially creating cell aggregates that could be deployed as a patch.

The most important example of hydrogel-based cardiac tissue engineering includes the work of Eschenhagen and colleagues. Cardiomyocytes were cast in growth factor–supplemented collagen gels and cultivated in the presence of cyclic mechanical stretch [13–16]. The main advantage of the hydrogel approach is the higher active force generated by such cardiac tissues, compared with the force generated by tissues on porous or fibrous 3D scaffolds. In addition, collagen and laminin are the main components of the myocardial ECM; thus, they are supportive of cardiomyocyte attachment and elongation. However, the main challenge remains in tailoring the shape and dimensions of such tissues. One interesting approach to addressing this issue is the use of extruded collagen type I tubes [17].

The use of porous scaffolds is another popular approach employed in cardiac tissue engineering. 3D cardiac tissue constructs were successfully cultivated in dishes using a variety of scaffolds, among which collagen sponges were the most common. In the pioneering approach of Li and colleagues, fetal rat ventricular cardiomyocytes were expanded after isolation, inoculated into collagen sponges, and cultivated in static dishes for up to 4 weeks [18]. The cells proliferated with time in culture and expressed multiple sarcomeres. Adult human ventricular cells were used in a similar system, although they exhibited no proliferation [19]. In another study, fetal cardiac cells were cultivated on porous alginate scaffolds in static 96-well plates. After 4 days of culture, the cells formed spontaneously beating aggregates in the scaffold pores [20]. Cell seeding densities on the order of 10^8 cells/cm^3 were achieved in the alginate scaffolds using centrifugal forces during seeding [21]. Neonatal rat cardiomyocytes formed spontaneously contracting constructs when inoculated in collagen sponges (Tissue Fleece) within 36 h after seeding [22] and maintained activity for up to 12 weeks. The contractile force increased upon addition of Ca^{2+} and epinephrine.

In a classical tissue engineering approach, fibrous polyglycolic acid (PGA) scaffolds were combined with neonatal rat cardiomyocytes and cultivated in spinner flasks and rotating vessels [23]. The scaffold was 97% porous and consisted of nonwoven PGA fibers 14 μm in diameter. This material has advantages from a clinical perspective because it is found in biodegradable sutures. Neonatal rat or embryonic chick ventricular myocytes were seeded onto PGA scaffolds by placing a dilute cell suspension in the spinner flasks and mixing for 3 days (50 rpm) [23]. Mixing in the spinner flasks (0, 50, or 90 rpm) had a significant effect on the construct metabolism and cellularity. Constructs cultivated in well-mixed flasks had a significantly higher cellularity index and metabolic activity compared with constructs cultivated in the static flasks. After 1 week of culture, constructs seeded with neonatal heart cells contained a peripheral tissue-like region (50–70 μm thick) in which cells stained positive for tropomyosin and organized in multiple layers in a 3D configuration [24]. Electrophysiological studies conducted using a linear array of extracellular electrodes showed that the peripheral layer of the constructs exhibited relatively homogeneous electrical properties and sustained macroscopically continuous impulse propagation on a centimeter-size scale [24]. Constructs based on the cardiomyocytes enriched by preparing in a well-mixed flask exhibited a lower excitation threshold (ET), higher conduction velocity, higher maximum capture

rate (MCR), and higher maximum and average amplitude of contraction. Laminar flow conditions in rotating bioreactors further improved the PGA-based constructs. The cells in the peripheral layer expressed tropomyosin and had a spatial distribution of connexin-43 comparable to that of the neonatal rat ventricle. The expression levels of cardiac proteins connexin-43, creatine kinase-MM, and sarcomeric myosin heavy chain were lower in rotating bioreactor cultivated constructs compared with the neonatal rat ventricle but higher than in the spinner flask–cultivated constructs [25]. In both spinner flasks and rotating bioreactors, the center of the constructs was mostly acellular owing to the oxygen diffusional limitations.

Electrospun scaffolds have gained significant attention because they enable control over the structure at submicron levels as well as over mechanical properties, both of which are important for cell attachment and contractile function. Microsized to nanosized fibers are created by electrically charging a stream of polymer that is then collected as a network of fibers. Electrospun fibers have a 3D fibrous structure and a high surface area to volume ratio that allows them to mimic the native ECM closely [26]. Another attractive property of electrospinning is the ability to make both random and highly aligned fibers. In one study, random poly(glycerol-sebacate) (PGS) and gelatin composite fibers were fabricated by collecting the fibers from a charged stream of polymer on a glass slide on a collector plate, whereas aligned fibers were collected on aluminum parallel electrodes (Fig. 61.2A). The tunable fiber alignment was demonstrated with different PGS–gelatin ratios and the microstructure was able to direct neonatal rat cardiomyocyte alignment, thus demonstrating the relationship between scaffold structure and cell morphology (Fig. 61.2B). Furthermore, the different fiber alignment and compositions possessed different mechanical properties, which were further shown to influence the contractile behavior of the cells, which allowed them to conclude that softer, more aligned fiber mats resulted in more synchronous beating [27].

The electrospinning protocol has been optimized for a wide range of biomaterials. Entcheva and colleagues [28] used electrospinning to fabricate oriented biodegradable, nonwoven poly(lactide) (PLA) scaffolds. Neonatal rat cardiomyocytes cultivated on oriented PLA matrices had remarkably well-developed contractile apparatuses and exhibited electrical activity. In a study by Parker and colleagues, a method known as rotary jet spinning, which allows for the fabrication of highly aligned fibers, was optimized for a variety of biomaterials. The polymer is fed through a syringe pump and collected on a rotating cylindrical collector. The ease of fabrication and resulting aligned fibers make this technique promising [29,30].

One hurdle that must be overcome in the area of electrospun scaffolds is the transport of oxygen and nutrients. Hasirci and colleagues attempted to overcome this by wrapping a biodegradable polyester electrospun aligned mat around a porous biodegradable tube composed of poly(L,D-lactide)lactic acid–PGS (Fig. 61.2C). The mat was cultured with mesenchymal stem cells before formation of the vascular construct and the tubing was perfused with cell culture media. This preliminary study can be used to develop thicker patches that can be integrated later with the existing vasculature of the heart [31].

Another common cardiac tissue engineering technique is to combine the benefits of the naturally occurring ECM and the stability of a porous scaffold. Freed and colleagues reported on the mechanical stimulation of hybrid cardiac grafts based on knitted hyaluronic acid–based fabric and fibrin [32]. The grafts exhibited mechanical properties comparable to those of native neonatal rat hearts. In a subcutaneous rat implantation model, the constructs exhibited the presence of cardiomyocytes and blood vessel ingrowth after 3 weeks. Another group inoculated collagen sponges or synthetic PGS scaffolds using Matrigel with neonatal rat cardiomyocytes [33]. The main advantage of a collagen sponge is that it supports cell attachment and differentiation. However, the scaffold tends to swell when placed in culture medium; therefore, the creation of parallel channel arrays resembling a capillary network is difficult. For that purpose, a biodegradable elastomer [34] with a high degree of flexibility was used.

Cardiac patches based on synthetic scaffolds are becoming highly sophisticated. They are able to stimulate and record electrical activity of the cardiac tissues relying on built-in electrodes. Dvir and colleagues engineered an electronic cardiac patch composed of gold electrodes embedded in a thin SU-8 mesh. An electroactive polymer capable of releasing proteins or small molecules upon electrical stimulation was loaded onto select electrodes. This sensor was then deposited with electrospun polycaprolactone-gelatin fibers, thus creating an electrospun biomaterial-electrode patch (Fig. 61.2D). The patch was then seeded with neonatal rat cardiomyocytes and cardiac FBs (Fig. 61.2E). They revealed that their device was able to support a homogeneous distribution of cells that interacted with the electronic fibers. Finally, an important function of an electronic patch is the ability to control the tissue remotely. Application of an electrical signal allowed the group to manipulate the direction of tissue contraction and propagation. Techniques that combine the fields of electronic sensors and biomaterials present an interesting approach in the attempt to develop, monitor, and control cardiac tissue [35].

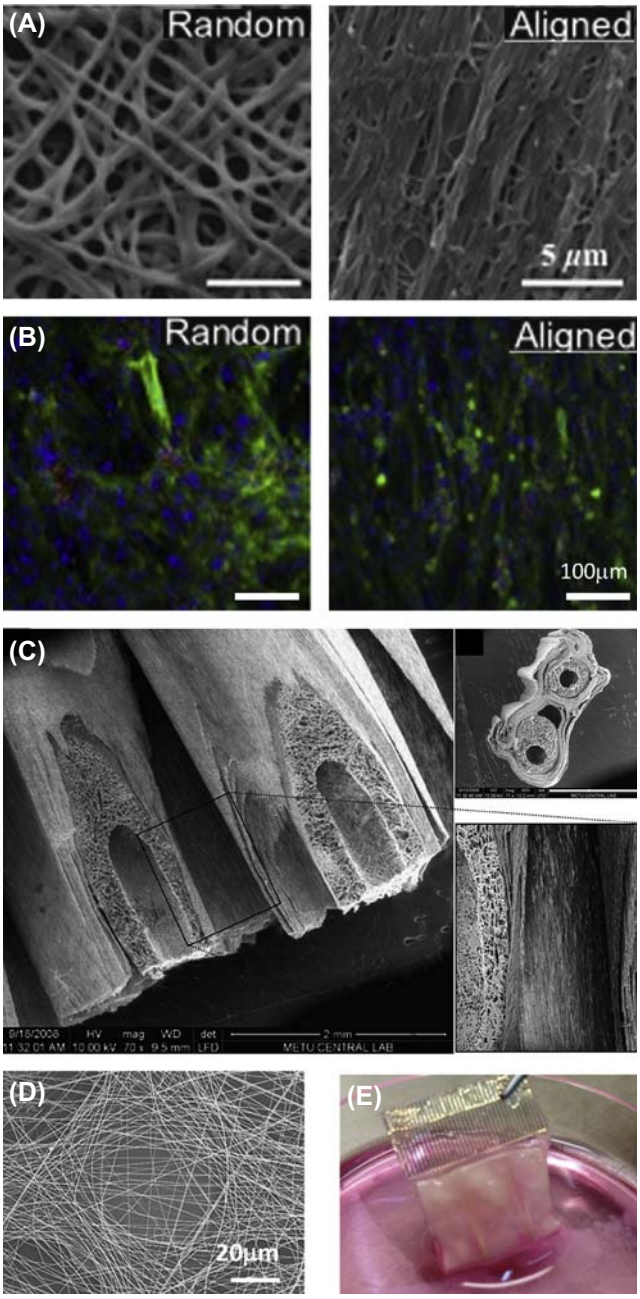

FIGURE 61.2 **Representative figure of electrospun patches in cardiac tissue engineering.** (A) Scanning electron micrograph of random and aligned electrospun polymer mats composed of poly(glycerol-sebacate) (PGS) and gelatin [27]. (B) Immunostaining for sarcomeric α-actinin (*green*), Cx-43 (*red*) and 4′,6-diamidine-2′-phenylindole dihydrochloride (*blue*) of neonatal rat cardiomyocytes seeded on the PGS—gelatin electrospun scaffolds in (A) [27]. (C) Scanning electron micrograph of an electrospun mat wrapped around a porous vascular tube [31]. (D) Electrospun polycaprolactone—gelatin fibers deposited on an electrode to form a biomaterial electronic hybrid [35]. (E) Image of a biomaterial electronic hybrid cultured for 7 days with neonatal rat cardiomyocytes and cardiac fibroblasts [35].

BIOPRINTING

3D bioprinting is a technique that has quickly gained popularity for its ability to create highly aligned tissues. Biopolymers can be encapsulated with cells and printed into lattice structures for the analysis for cardiac function. A technique that can potentially combine the advantages of the hydrogel approach with ease in tailoring tissue shape and size is inkjet printing. Cardiac constructs based on feline cardiomyocytes were created by printing cell

solutions onto alginate and using calcium as a cross-linking agent [36]. This approach may be particularly useful for coculture because it enables precise control over cell location in the tissue construct.

Pati et al. developed a novel decellularized ECM bioink capable of printing various tissues. The ink, which consists of decellularized heart tissue, had a positive effect on cardiac tissue maturation. They demonstrated that there was an upregulation of fast myosin heavy chain and α-sarcomeric actinin compared with the control gel [37]. Alternatively, a gelatin-based bioink encapsulated with gold nanoparticles was developed by Zhu et al. (Fig. 61.3A). The conductive bioink was loaded with neonatal rat cardiomyocytes and was printed into a lattice structure. The cardiac scaffolds showed synchronous beating as early as day 2 of culture. Immunostaining showed increased connexin-43 gap junction proteins and sarcomeric structures, which suggested that the conductive bioink aided in tissue maturation (Fig. 61.3B) [38].

Another bioprinting approach consists of printing a mosaic hydrogel sheet. The sheet is composed of alternating bioinks containing cells and can be printed in an aligned fashion to mimic the heart's structure (Fig. 61.3C). This method will allow for design in cell alignment as well as the controlled incorporation of other cell types. This method

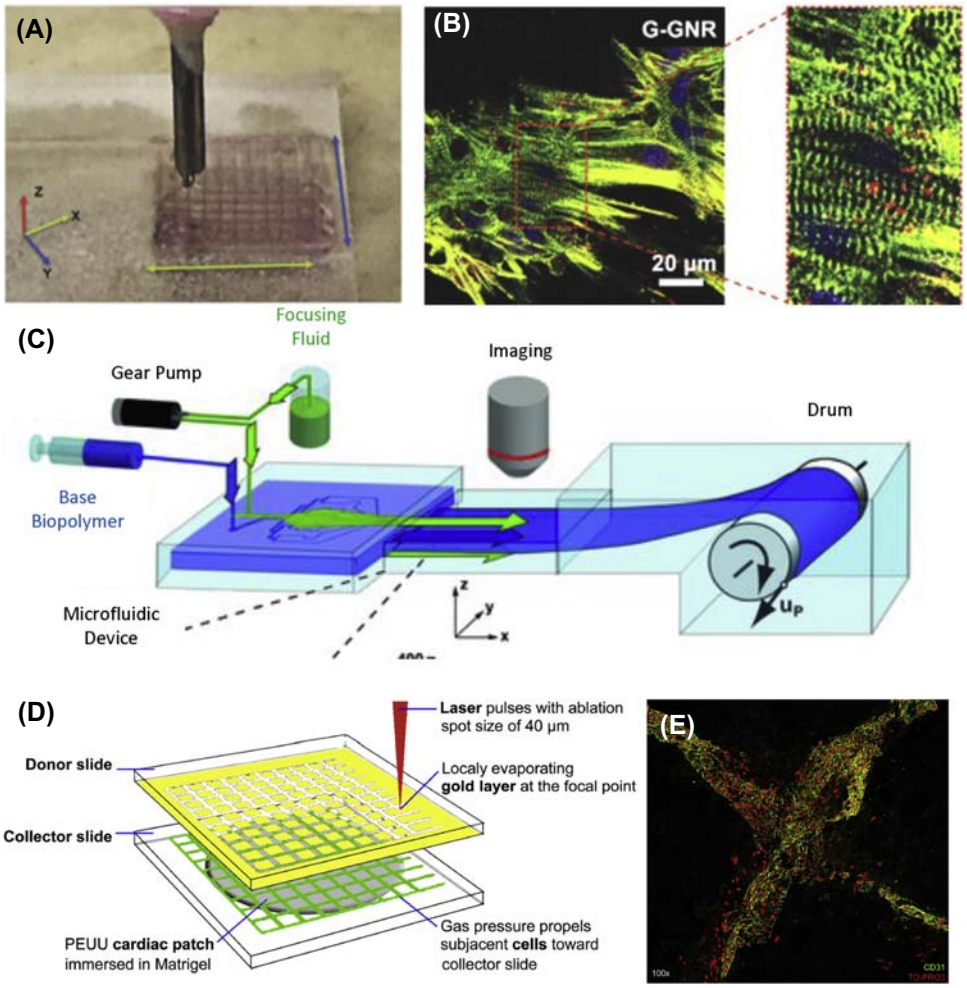

FIGURE 61.3 Applications of bioprinting to create anisotropic and vascularized scaffolds. (A) Image of an extrusion needle printing a gold nanoparticle gelatin methacryloyl construct embedded with neonatal rat cardiomyocytes and cardiac fibroblasts [38]. (B) Immunostaining of neonatal rat cardiomyocytes embedded in a gold nanoparticle gelatin methacryloyl three-dimensionally (3D) bioprinted construct for sarcomeric α-actinin (*green*), Cx-43 (*red*) and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (*blue*) [38]. (C) Schematic of a bioprinting approach that can be used to print a mosaic hydrogel sheet. Using microfluidics, a base polymer is extruded for a flat sheet. This method can be modified to print numerous polymer compositions simultaneously [39]. (D) Laser-induced forward-transfer cell printing capable of printing human umbilical vein endothelial cell (HUVEC)-laden patches [41]. (E) Immunostained vascularized patch stained for CD31 (*green*) and TO-PRO3 (*red*) [41].

is still fairly new; further optimization is required to increase the resolution. However, it presents a novel approach in the development of a full-sheet cardiac patch [39].

Incorporation of human iPSC-derived cardiomyocytes into any cardiac platform is a desirable property; however, the shear stresses typically associated with bioprinting increase the complexity of such a task. Therefore, a method is needed that can accurately print a structure similar to the native ECM. Zhang and colleagues developed the method of multiphoton-excited 3D printing that enabled them to generate an ECM-mimicking construct. The scaffold was generated by depositing gelatin methacrylate in a grid-shaped pattern modeled after the native deposition of fibronectin. Human iPSCs were differentiated into cardiomyocytes, smooth muscle cells, and ECs and seeded on the bioprinted scaffold. The bioprinted patches were then engrafted into a murine model of MI. The patch was shown to improve cardiac function of the treated animals; however, further work is required to confirm electromechanical integration of the patch [40].

In addition to creating biomimetic scaffolds, bioprinting can be used to create vascularized patches. The human umbilical vein endothelial cell (HUVEC)-laden patch was created through laser-induced forward-transfer cell printing (Fig. 61.3D). Briefly, HUVEC cells were seeded on a prepatterned polyurethane cardiac patch that was deposited onto a collector plate by short laser pulses (Fig. 61.3E). The patch was implanted in an MI rat model and rats with the HUVEC-laden patches showed significantly improved heart function. In addition, the vascularized patches resulted in a higher capillary density around the infarct area suggesting vascular integration [41].

Therefore, many techniques have arisen in the field of cardiac tissue engineering. Each technique has its own advantage and disadvantage, but there seems to be a general trend of increasing complexity in an effort model the native cardiac environment to more accurately.

CARDIAC ORGANOIDS AND ORGAN-ON-A-CHIP ENGINEERING

Cardiac organoids, microphysiological systems, and organ-on-chip platforms have become popular for their ability to recapitulate properties found in the native tissue. These devices are sought as models in drug toxicity studies and disease modeling. In early work, contractile organoids 24 mm long and 100 μm thick were fabricated by self-organization [42]. Cardiomyocytes were cultivated on a polydimethylsiloxane (PDMS) surface coated with laminin. As laminin degraded, the confluent monolayer detached from the periphery of the substrate, moving toward the center and wrapping around a string placed in the center of the plate until a cylindrical contractile organoid was formed.

A significant step toward a clinically useful cardiac patch was the cultivation of ESC-derived cardiomyocytes on thin polyurethane films. Cells exhibited cardiac markers (actinin) and were capable of synchronous macroscopic contractions [43]. The orientation and cell phenotype could be improved further by microcontact printing of ECM components (e.g., laminin), as demonstrated for neonatal rat cardiomyocytes cultivated on thin polyurethane and PLA films [44,45].

We have used microfluidic patterning of hyaluronic acid on glass substrates to create thin (10- to 15—μm-diameter), several millimeter−long cardiac organoids that exhibited spontaneous contractions and stained positive for troponin I, a cardiac marker [46].

Feinberg et al. seeded a layer of neonatal rat ventricular cardiomyocytes on a PDMS membrane that could be detached from a thermosensitive poly(isopropylacrylamide) layer at room temperature. Called muscular thin films, these cell-covered sheets could be designed to perform tasks such as gripping, pumping, walking, and swimming by careful tailoring of the tissue architecture, thin-film shape, and electrical-pacing protocol [47].

Scaffold structure can be used to guide the orientation of cardiomyocytes effectively and yield an anisotropic structure similar to the native myocardium even in the absence of specific physical cues such as electrical or mechanical stimulation. Freed and colleagues created an accordion-like scaffold using laser boring of a 250-μm-thick PGS layer [48]. The accordion-like honeycomb was designed by overlapping two 200 × 200-μm squares at an angle of 45 degrees. The pore walls and struts were about 50 μm thick. The scaffolds were pretreated with cardiac FBs followed by the seeding of enriched cardiomyocytes. During pretreatment, rotating culture was used, whereas static culture was used upon cardiomyocyte seeding. At the end of cultivation, the authors obtained contractile cardiac grafts with mechanical properties closely resembling those of the native rat right ventricle. In addition, the cells in the pores were aligned along the preferred direction.

Badie et al. investigated yet another method to replicate the microstructure of heart tissue in vitro. The two-step method first involves imaging the heart using diffusion tensor magnetic resonance imaging (DTMRI). From the 3D reconstructed image, a specific 2D plane is chosen and the cardiac fiber directions on this plane are converted into soft-lithography photomasks, and later into fibronectin-coated PDMS sheets. Fibronectin patterns consisted of a matrix of 190-μm^2 subregions, each composed of parallel lines 11–20 μm wide, spaced 2–8.5 μm apart, and angled to match local DTMRI-measured fiber directions. By adjusting fibronectin line widths and spacing, cell elongation, gap junctional membrane distribution, and local cellular disarray were altered without affecting the cell direction. This approach enabled the systematic studies of intramural structure–function relationships in both healthy and structurally remodeled hearts [49,50].

Bursac and colleagues developed a cell–fibrin hydrogel micromolding approach in which PDMS molds containing an array of elongated posts were used to fabricate relatively large neonatal rat skeletal muscle tissue networks. As the cells compacted in the hydrogel, the presence of high–aspect ratio posts forced them to elongate and align, thus imparting a high degree of anisotropy to the cells and the tissue. This approach is being extended to the cultivation of cardiac patches based on mouse ESC-derived progenitor cells [51,52].

Interestingly, a high degree of anisotropy correlating with the high propagation velocities in the longitudinal direction (~35 cm/s) was achieved by cultivation of neonatal rat cardiomyocytes of micromolded poly(ethylene glycol) hydrogels with submicrometer features, specifically alternating 800 × 800-nm grooves and ridges. The submicrometer features forced the cells to align focal adhesions along the groove and ridge direction and the cytoskeleton followed [53].

Typical culture platforms are limited in their ability to develop mature tissue, because iPSC/ESC-derived cardiomyocytes are immature after differentiation, and therefore are limited in their ability to truly model the cardiac environment. Another prominent heart-on-a-chip cardiac model is called the Biowire (Fig. 61.4A). The Biowire platform combines electrical and architectural cues, thus creating a microenvironment capable of fostering mature cardiac tissue. The platform consists of human ESC (hESC)-derived cardiomyocytes seeded in a collagen gel around a surgical suture, which is embedded in a PDMS template. Throughout culture, the cardiomyocytes and collagen gel compacted around the wire, forming a dense tissue. The tissue was electrically stimulated for 7 days and had highly organized sarcomeric banding, which suggested maturation of the tissue (Fig. 61.4B). Stimulation during culture was also shown to improve Ca^{2+} influx and the electrophysiological properties, which further supported the hypothesis of improved tissue maturation upon electrical stimulation. Future iterations of the model should support the use of vascular cells to aid in oxygen and nutrient diffusion [54]. The platform was later modified to support seeding around a polytetrafluoroethylene tube. The tubing can be perfused with various drugs and the surrounding tissue can be monitored to test for cardiotoxicity [55].

Cardiac organoids and organ-on-a-chip devices can also be used for disease modeling applications. A model aims to study diseased myocardium by creating arrays of ventricular tissue subjected to cyclic stress. Their goal was to induce diseased and failing myocardium by mimicking mechanical overload. The chip consisted of flexible micropatterned substrates placed inside a chamber ring that stretched the samples to 10% strain by a custom system. The group found that they were able to induce myocardium failure by studying genetic, structural, and function response, suggesting a novel platform to study the response of diseased tissue. In future studies, they hope to extend the platform to include drug toxicity testing [56].

Another model attempts to reproduce the hallmarks of cardiomyopathy associated with the mitochondrial disease Barth syndrome. Barth syndrome is the result of a gene mutation that results in abnormalities in the sarcomeric assembly and contraction of the heart. Using iPSCs from patients with Barth syndrome, cells were seeded onto micropatterned fibronectin sheets to examine sarcomeric organization. The sarcomeric structure of the Barth syndrome cells was incomplete and sparse compared with healthy control cells. This allowed the group to confirm that sarcomeric organization depends on proper functioning of the mitochondria. To characterize the contractile response of the tissue, the cells were integrated into their heart-on-a-chip model, which consists of a flexible PDMS cantilever; this allowed the researchers to confirm impaired cell contractility (Fig. 61.4C). Once validated, the model was used to evaluate the effect of possible therapies, thus proving the effectiveness and need of disease models for therapeutic testing [57].

In another effort to model dilated cardiomyopathy, Chen and colleagues attempted to determine how a genetic mutation affects the sarcomeric protein, titin. Human iPSC-derived cardiomyocytes were seeded onto microarray post detectors, which allow for robust observation of contractile activity. Unsurprisingly, sarcomeres were not

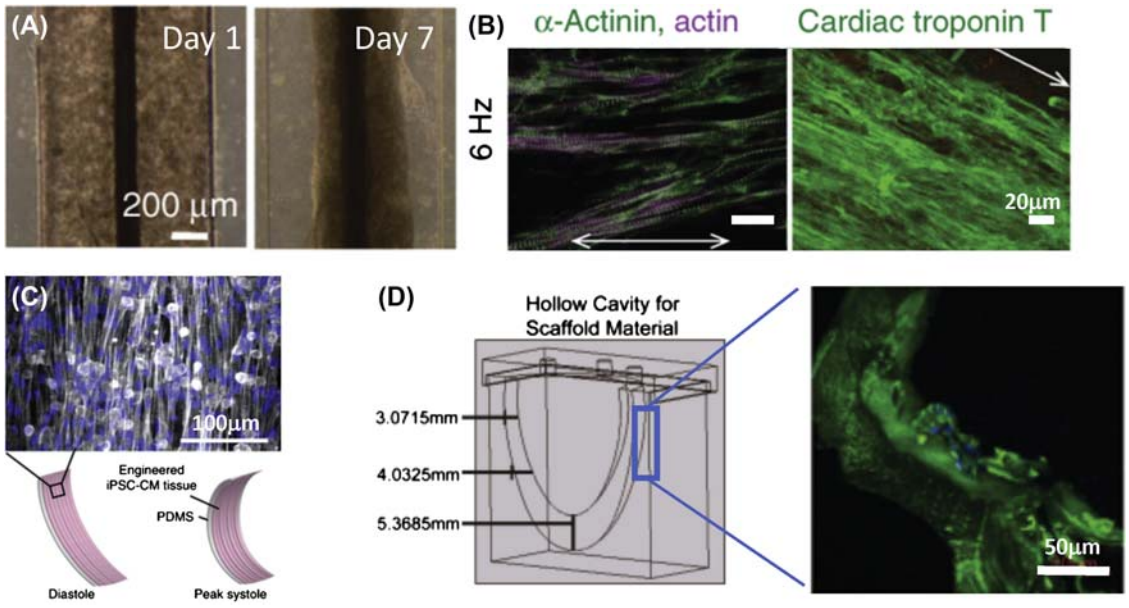

FIGURE 61.4 **Cardiac organoids and heart-on-a-chip engineering.** (A) Microscopic image of a heart-on-a-chip platform called the Biowire showing the contraction of human embryonic stem cell (ESC)-derived cardiomyocytes and cardiac fibroblasts contracting over 7 days [54]. (B) Immunostaining of human ESC-derived cardiomyocytes and cardiac fibroblasts after 7 days of electrical stimulation at 6 Hz showing strong cardiac specific marker expression and sarcomeric formation [54]. (C) Organ-on-a-chip cantilever platform used to model Barth syndrome, a mitochondrial disease (bottom). The platform is patterned with fibronectin lines to allow for anisotropic organization. Sample has been immunostained for sarcomeric α-actinin (*white*) and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (*blue*) [57]. (D) An approach using three-dimensional (3D) printing to develop a mold modeled after the left ventricle. The mold is injected with chitosan and lyophilized before seeding. Immunostaining (right) shows poor spreading and clustering of cells. Sarcomeric α-actinin (*green*), and DAPI (*blue*) [62,63].

only shorter but more disorganized in titin-deficient samples. Furthermore, the model was used to identify other missense variances that resulted in a mutated titin protein. This approach can be used for further gene expression modification of not only titin but also other cardiac proteins to model various diseases [58].

A universally accepted cardiac model cultured in serum-free media is necessary in the field for disease modeling and the pharmaceutical industry. Zimmermann and colleagues developed a proof-of-concept system that could be scaled up and used in applications ranging from disease modeling to a cardiac patch. Using a 1:1 ratio of human iPSC-derived cardiomyocytes and nonmyocytes, the cells are seeded around a cantilever post structure. The cells are cultured in serum-free media, thus complying with Current Good Manufacturing Practice standards. This platform was found to foster the maturation of the cells better as opposed to a conventional monolayer. Finally, in an effort to demonstrate the scalability of the platform, the cardiomyocytes and nonmyocytes were seeded around a custom array of posts. The array of posts supported the formation of a functional tissue sheet that, when sutured over the epicardium of a rat heart, exhibited signs of vascularization. Such a platform, as described earlier, should be taken only as a proof-of-concept model. Further work is required to develop standardized models that support disease modeling and tissue repair [59].

One major limitation to heart-on-a-chip platforms is evaluation of tissue functionality, because a main means of measuring tissue response is by microscopy and measuring the amplitude and frequency of the beating tissue. An alternative approach consists of combining sensors in the microphysiological system for continuous monitoring. Lind et al. combined 3D printing and microphysiological systems to develop a platform capable of tissue contractile stress. They developed six inks based on piezoresistive, high-conductance, and biocompatible soft materials. This chip, which is printed using these materials, consists of a cantilever system with micropatterns on top to direct cell alignment. The microphysiological system provided long-term, continuous data acquisition, thus proving its usefulness for drug studies [60].

ENGINEERING THE ENTIRE VENTRICLE

Using a collagen-based hydrogel, Lee et al. developed an in vitro ventricle chamber. Briefly, isolated neonatal rat cardiomyocytes were suspended in collagen and pipetted into a cup-shaped mold around a balloon catheter. After collagen gel polymerization, the organoid was cultured in cell media until testing. Their approach allowed them to create a 3D cardiac encapsulated ventricle chamber that was able to contract spontaneously and expel a measurable volume. However, they noted that the cells maintained an immature and random orientation, unlike the aligned anisotropy found in native heart tissue [61].

Another cardiac ventricle, developed by Patel et al., consists of a ventricle-shaped chitosan tissue formed by freezing chitosan in a 3D printed computer-aided design (CAD) model of the left ventricle (Fig. 61.4D). The chitosan was then lyophilized and rehydrated before seeding. Neonatal rat cardiomyocytes were injected intermittently throughout the scaffold and a cardiac seeded patch was wrapped around the outside. However, there are considerable differences between the scaffolds and the CAD models, and the injected cells remained in clusters and failed to spread throughout the scaffold. Further work is required before the platform can be used to examine the pressure–volume response of the cardiac ventricle [62,63].

BIOREACTORS AND CONDITIONING

Major efforts in the development of bioreactors for tissue engineering of myocardium focus on (1) providing sufficient oxygen supply for the highly metabolically active cardiomyocytes, and (2) providing appropriate physical stimuli necessary to reproduce a complex structure at various length scales (subcellular to tissue).

The most common culture vessels used for tissue engineering of the myocardium include static or mixed dishes, static or mixed flasks, and rotating vessels. These bioreactors offer three distinct flow conditions (static, turbulent, and laminar) and therefore differ significantly in the rate of oxygen supply to the surface of the tissue construct. Oxygen transport is an important factor for myocardial tissue engineering owing to the high cell density, limited cell proliferation, and low tolerance of cardiac myocytes for hypoxia. In all configurations, oxygen is supplied only by diffusion from the surface to the interior of the tissue construct, yielding an approximately 100-μm-thick surface layer of compact tissue capable of electrical signal propagation and an acellular interior [64].

Oxygen Supply

In an attempt to enhance mass transport within cultured constructs, a perfusion bioreactor that provides interstitial medium flow through the cultured construct at velocities similar to those found in the native myocardium (~400–500 μm/s) was developed [65]. In such a system oxygen and nutrients were supplied to the construct interior by both diffusion and convection (Fig. 61.5A). Interstitial flow of culture medium through the central 5 mm–diameter × 1.5 mm–thick region resulted in physiologic dense, viable, differentiated, and aerobically metabolizing cells. In response to electrical stimulation, perfused constructs contracted synchronously, had a lower ET, and recovered their baseline function levels of ET and MCR after treatment with a gap junctional blocker. On the other hand, dish-grown constructs exhibited arrhythmic contractile patterns and failed to recover their baseline MCR levels. These studies suggested that the immediate establishment and maintenance of interstitial medium flow markedly enhanced the control of oxygen supply to the cells and thereby enabled engineering of compact constructs. However, most cells in perfused constructs were round and mononucleated, indicating that some of the regulatory signals (either molecular or physical) were not present in the culture environment.

In another approach, a separate compartment for medium flow was created by perfusing channeled scaffolds in a configuration resembling the capillary network in vivo. Neonatal rat heart cells were inoculated into the pores of an elastic, highly porous scaffold (PGS) with a parallel channel array and perfused with a synthetic oxygen carrier (Oxygent in culture medium, perfluorocarbon [PFC] emulsion) [33]. Constructs cultivated with PFC emulsion had significantly higher DNA content, significantly lower ET, and a higher relative presence of cardiac markers troponin I and connexin-43 (Western blot) compared with the culture medium alone. Cells were present throughout the construct volume. In this configuration, the presence of PFC emulsion further enhanced the

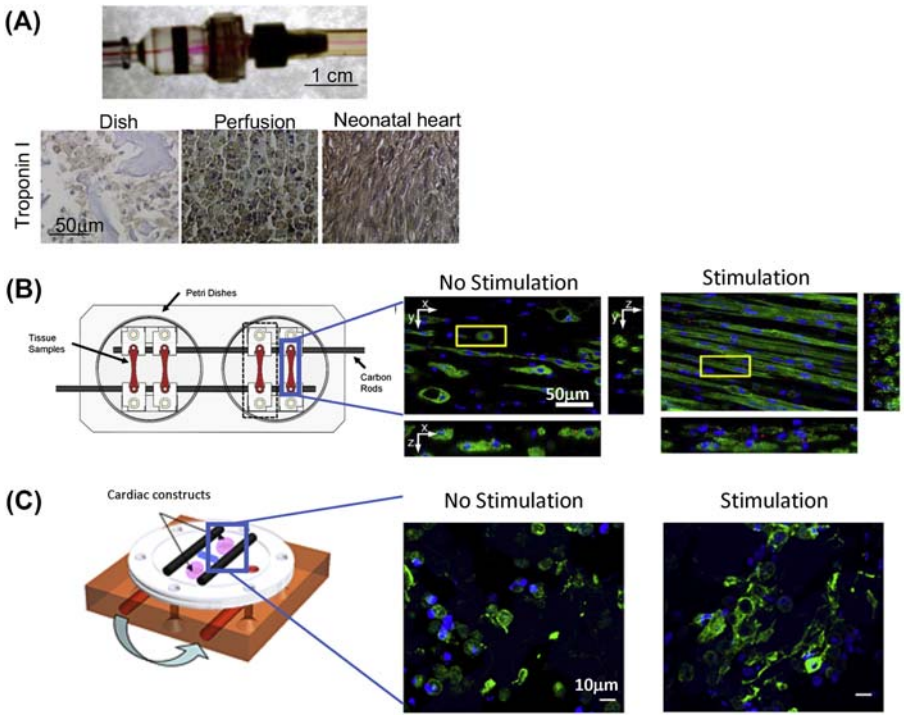

FIGURE 61.5 **Cardiac tissue engineering culture systems.** (A) Direct culture medium perfusion of constructs based on neonatal rat cardiomyocytes inoculated into collagen sponges using Matrigel. Medium perfusion resulted in uniform cell distribution and maintenance of cell viability. Immunohistochemical staining illustrated cross-sectional distribution of cells expressing cardiac troponin I [73]. (B) Bioreactor designed to stimulate the sample. Immunostaining for sarcomeric α-actinin (*green*), Cx-43 (red), and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (*blue*) reveals elongated sarcomeric structures and expression of Cx-43 similar to the native adult myocardium [76]. (C) Hybrid bioreactor designed to electrically stimulate the samples and subject the constructs to a flow rate of 0.1 mm/s. The stimulated samples were more elongated and had a stronger expression of troponin I (green) [77].

oxygen supply to the cells by improving both axial (convective term) and radial (effective diffusivity) transport properties [64].

Kofidis et al. supplied pulsatile flow to cardiomyocytes encapsulated in fibrin glue around a rat artery in vitro [66]. Dvir et al. designed a novel perfusion bioreactor that employs a distributing mesh upstream from the construct to provide homogeneous fluid flow and maximum exposure to perfusing medium [67]. This convective supply of oxygen led to increased cell viability in alginate scaffolds seeded with physiologically relevant cells [67]. In addition, pulsatile culture medium flow resulted in physiological cardiac hypertrophy via stimulation of the Erk pathway [67,68]. We combined mechanical stimulation and perfusion in a single system by using a normally closed pinch valve at the outlet from the perfusion chamber. The valve was set to open at the frequency of 1 Hz. The buildup of the culture medium during the closed period resulted in tissue compression followed by relaxation at the valve opening [69].

Differentiation

Mechanical Stimulation

One significant approach to cardiac tissue engineering, established by Eschenhagen and colleagues [13,14,16,70], involves cultivating neonatal rat heart cells in collagen gel or Matrigel in the presence of growth factors. The cultured tissues are subjected to sustained mechanical strain. Under these conditions, cardiomyocytes and nonmyocytes form 3D cardiac organoids, consisting of a well-organized and highly differentiated cardiac muscle syncytium that exhibited contractile and electrophysiological properties of working myocardium. First

implantation experiments in healthy rats showed survival, strong vascularization, and signs of terminal differentiation of cardiac tissue grafts [15].

In the state-of-the art approach by Eschenhagen and colleagues, neonatal rat cardiac cells were suspended in the collagen−Matrigel mixture and cast into circular molds [16]. After 7 days of static culture, the strips of cardiac tissue were placed around two rods of a custom-made mechanical stretcher and subjected to either unidirectional or cyclic stretch. Histology and immunohistochemistry revealed the formation of intensively interconnected, longitudinally oriented cardiac muscle bundles with morphological features resembling adult rather than immature native tissue. Primitive capillary structures were also detected. Cardiomyocytes exhibited well-developed ultrastructural features: sarcomeres arranged in myofibrils, with well-developed Z, I, A H and M bands, specialized cell−cell junctions, T tubules, as well as well-developed basement membranes. Contractile properties were similar to those measured for native tissue, with a high ratio of twitch to resting tension and strong β-adrenergic response. The action potential characteristics of rat ventricular myocytes were recorded.

Another way mechanical stimulation assists in tissue maturation is through improved orientation of gap junction proteins. Connexin 43 is a gap junction protein specific to cardiac tissue and is responsible for intercellular communication. In a study led by Dhein and colleagues, neonatal rat cardiomyocytes were cultured on silicon membranes that were subjected to uniaxial cyclic stretch for 24 h. The samples were stained for connexin-43 and other cardiac markers and the cells exposed to the mechanical stretch were elongated in morphology. Connexin 43 was found to be polarized transversely in the direction of cyclic stress [71].

Electrical Stimulation

In the native heart, mechanical stretch is induced by electrical signals. Contraction of the cardiac muscle is driven by the waves of electrical excitation (generated by pacing cells) that spread rapidly along the membranes of adjoining cardiac myocytes and trigger the release of calcium, which in turn stimulates contraction of the myofibrils. Electromechanical coupling of myocytes is crucial for their synchronous response to electrical pacing signals, resulting in contractile function and pumping of blood [72].

We prepared cardiac constructs by seeding collagen sponges with neonatal rat ventricular cells that were electrically stimulated using suprathreshold square biphasic pulses (2 ms in duration, 1 Hz, 5 V) [73]. The stimulation was initiated after 1−5 days of scaffold seeding (a 3-day period was optimal) and applied for up to 8 days. Over only 8 days in vitro, electrical field stimulation induced cell alignment and coupling, increased the amplitude of synchronous construct contractions by a factor of seven, and resulted in a remarkable level of ultrastructural organization. The development of conductive and contractile properties of the cardiac constructs was concurrent, with a strong dependence on the initiation and duration of the electrical stimulation. Aligned myofibers expressing cardiac markers were present in stimulated samples and neonatal heart. Stimulated samples had sarcomeres with clearly visible M and Z lines, and H, I, and A bands. In most cells, Z lines were aligned and the intercalated discs were positioned between two Z lines. Mitochondria (between myofibrils) and abundant glycogen were detected. In contrast, nonstimulated constructs had poorly developed cardiac-specific organelles and poor organization of ultrastructural features.

Subsequently, we applied biphasic electrical stimulation to mimic conditions in the heart further and demonstrated that electrical stimulation enhances the assembly of cardiac organoids based on multiple cell types, including FBs, ECs, and cardiomyocytes [74]. We also developed cardiac microchips that combine electrical field stimulation and topographical cues. Specifically, micrometer-sized grooves and ridges were created by hot-embossing polystyrene placed between gold electrodes on a single chip. Simultaneous application of topographical cues and electrical field stimulation resulted in a remarkable level of cardiomyocyte alignment, elongation, and assembly of contractile apparatus [75].

Further applying this work, Hitchcock and colleagues used electrical stimulation to engineer mature cardiac tissue, similar to a native phenotype. In the native heart myocardium, connexin-43 is concentrated at the end of cells; however, in unstimulated heart myocardium, connexin-43 is typically sporadically expressed, thus providing motivation for electrical stimulation. The authors developed a specialized bioreactor in which neonatal rat cardiomyocytes were seeded around two posts to form an engineering cardiac tissue fiber (Fig. 61.5B). After 3 days of culture, the samples were electrically stimulated by carbon electrodes. The group found that the stimulated samples were more elongated and had a stronger expression of sarcomeric structures, which was similar in morphology to the native heart myocardium. Connexin 43 expression was found to be significantly

increased in the stimulated sample compared with the unstimulated one and was expressed at levels similar to native adult myocardium [76].

In an attempt to mimic adult native myocardium more accurately, more complex bioreactors are being developed to stimulate the tissue. A combination bioreactor designed to stimulate electrically and provide biomimetic perfusion rates was developed by Vunjak-Novakovic and colleagues (Fig. 61.5C). Neonatal rat cardiomyocyte tissue constructs were subjected to a flow velocity of 0.1 mm/s and were stimulated with a 3-Hz monophasic square wave. The stimulated samples were the most abundant and were more elongated compared with unstimulated samples. A limitation of the study was the spread of neonatal cardiomyocytes. The group used PGS for the tissue scaffold, which does not readily support cell attachment. As a result, the cells appeared to cluster on the tissue. However, this study serves as an example of the advantages of mimicking environmental cues in cardiac tissue engineering [77].

Hence, the in vitro application of a single but important in vivo factor progressively enhanced the functional tissue assembly and improved the properties of engineered myocardium at the cellular, ultrastructural, and tissue levels.

TISSUE AND ORGAN FUNCTION

Successful implantation of engineered tissues requires both maintenance of the cellular phenotype and the functional integration of the construct within the host tissue. As progress is made from the state of the art described earlier to the final goal, it will be necessary to ensure that engineered cardiac cells and tissue contract in unison with the surrounding native myocardium to produce the desired force, but also that the graft is electrically integrated with the host to prevent arrhythmogenesis.

Underlying such integration and the implicit control of the construct phenotype is the creation of arborized networks (vessels, lymphatics, and nerves) needed to sustain large and complex tissue structures. Then there are issues associated with blood compatibility, tissue remodeling, and more generally immune and inflammatory responses to the new tissue or cells. Using autologous cells is an approach that is immunologically preferable, but it likely precludes the "off-the-shelf" concept behind much of the attraction of tissue engineering.

Mechanical Elasticity and Strength Development

A critical feature of a heart is its mechanical characteristics. Simply speaking, the heart must pump blood at a mean pressure of roughly 100 mmHg. Hence, heart muscle must stretch in response to capillary filling pressure and eject a volume of blood that varies with demand. The latter requires a uniform and well-coordinated contraction that generates the required power. The mechanical fatigue limitations of a heart that must beat 3×10^8 times over 10 years must be compared with the flexural fatigue life of synthetic elastomeric materials that are typically much lower.

It will be a significant challenge to replicate the complex architecture of the myocardium and its nonlinear viscoelastic properties in both resting and activated states [78]. Although some constructs exhibit a significant burst strength and some groups are advanced in the use of the tools of biomechanics to advance vascular graft [79] or heart valve development, this area has received less attention than it deserves [80].

Tissue Architecture and Electrical Conduction

The complexity of the electrical conduction pathways in the heart is just starting to gain attention in tissue engineering literature. The cells need to form the appropriate intercellular connections and matrix arrangements to enable the directed beating of contracting cells to generate the forces required to pump blood [81]. The proper formation of the intercalated disks between myocytes is also critical in enabling electrical pulses to be transmitted in the correct direction at normal speeds and in allowing suitable force transmission. The heart also contains specialized cells that participate in the electrical conduction routes found throughout the heart. These specialized cells are crucial to coordinating the heart's contractile effort; including them in the proper places in a regenerated substitute may be critical. There are clear differences between the rhythmic twitching of cultured cardiac cells en mass and the

organized, efficient, regulated beating of the heart; only the latter will generate the force required to pump blood at systolic pressure levels. It is not difficult to envision the problems yet to be faced. Given the variety of electrical conduction–related diseases in a normal myocardium, there is good reason to suspect that simple mimicry of heart muscle may fall short of the goal. The protocol for directed differentiation of human pluripotent stem cells, specifically hESCs and iPSCs, has sufficiently advanced to enable differentiation of preferentially ventricular cells [82] as well as more specialized cells of the conduction system, e.g., sinoatrial nodal cardiomyocytes [83]. These advances are paving the way to engineering cardiac tissues with specialized cardiomyocyte subtypes to reproduce the complexity of the native tissue composition.

Thrombogenicity and Endothelialization

The need for blood compatibility is another crucial characteristic of cardiovascular constructs. All biomaterials lack the desired nonthrombogenicity and most ECMs initiate thrombosis; hence, endothelialization of the construct is another critical issue. ECs have a reversible plasticity [84–86] and can become activated (proliferative or adhesive to leukocytes) upon exposure to inflammatory cytokines (e.g., interleukin-1 [IL-1], tumor necrosis factor [TNF]) or to growth factors such as vascular endothelial growth factor (VEGF). Flow and the associated shear stress, normally in the range of 5–20 dyn/cm^2, elongate and align cells in the direction of flow [87,88] and modify gene expression [89] as well as many other functions including markers of antithrombogenicity.

ECs provide a hemocompatible surface by producing molecules that modulate platelet aggregation (e.g., prostacyclin), coagulation (thrombomodulin [90,91]), and fibrinolysis [92] (e.g., tissue plasminogen activator). They can be transformed into a prothrombotic surface: for example, by the action of thrombin or through exposure to some biomaterials [93–96]. Blood compatibility has been an important issue in the development of vascular grafts. Clinical success [97] has renewed enthusiasm for seeding grafts with ECs. In some protocols, many of the preseeded cells are lost upon implantation owing to insufficient adhesion [98], and thus protection from thrombosis provided by the cells is limited because of incomplete cell coverage. The potential to exploit the presence of circulating EC progenitors has only begun to be explored [99].

It is also worth noting the effects of the endothelium on the neighboring tissue and the corresponding effects on EC phenotype. With vascular smooth muscle cells (VSMCs), this bidirectional cross-talk is thought to be a critical regulator of vascular homeostasis [100]: Secretion and expression of molecules such as nitric oxide [101], prostacyclin [102], and endothelin [103] act on VSMCs to regulate vessel tone. Meanwhile, VSMCs inhibit EC endothelin 1 production to increase EC nitric oxide and endothelial nitric oxide synthase expression [104]. Many other relevant systems (e.g., matrix metalloproteinase [MMP] secretion and matrix remodeling) are also affected by the interactions between EC and other cell types. Moreover, VEGF inhibits pericyte coverage under conditions of PDGF-mediated angiogenesis [105], complicating vascularization strategies built around VEGF delivery (see subsequent discussion).

Vascularization

The intrinsic nature of large cell-based constructs and the corresponding difficulty of supplying cells deep within the construct with nutrients are yet other problems. Diffusion is fine for 100 μm or so and low cell densities can extend this limit, but at the cost of making constructs too large to be useful. Thin or essentially 2D constructs (e.g., a tube) are feasible without an internal blood or nutrient supply. However, it is hard to combine cells at tissue densities ($>10^8$ cells/cm^3) into large tissues without some sort of prevascularization or its alternative. Thus, a capillary network (and a lymphatic network) needs to be "engineered" as part of the creation of a larger structure.

In a cell-free approach, vascularization and improvement of left ventricular (LV) function after MI were achieved by the sustained release of basic fibroblast growth factor (bFGF) incorporated into gelatin microspheres [106], acidic fibroblast growth factor (FGF) from ethylene vinyl acetate copolymer [107], and bFGF from heparin-alginate beads [108]. Mooney and colleagues incorporated an endothelial cell mitogen (VEGF) into 3D porous poly(lactide-*co*-glycolide) scaffolds during fabrication [109] to promote scaffold vascularization. Sustained delivery of bioactive VEGF translated into a significant increase in blood vessel ingrowth in mice and the vessels appeared to integrate with the host vasculature. We use microencapsulated $VEGF_{165}$-secreting cells (prepared

by transfection of L929 cells) as a means of exploring this strategy, at least for microcapsules [110]. Of course, VEGF is but one angiogenic factor [111] and issues associated with the functional maturity of the vessels and the need for multiple factors may limit this strategy. In a third approach, Vacanti et al. micromachined a hierarchical branched network mimicking the vascular system in 2D. Silicon and Pyrex surfaces were etched with branching channels ranging from 500 to 10 μm in diameter [112] that were then seeded with rat hepatocytes and microvascular ECs.

We covalently immobilized VEGF165 and angiopoietin-1 to porous collagen scaffolds to enhance scaffold vascularization in vitro and in vivo [113]. Such covalent immobilization offers the advantages of prolonged signaling and lowers the total amount of growth factors required; it also offers the possibility of generating capillary-like structures in the tissue engineered scaffolds in vitro.

A prevascularized skeletal muscle was created [114] by coculturing skeletal muscle cells with ESC-derived EC and FBs. It appeared that up to 40% of the engineered blood vessels "connected" to the host vasculature upon implantation in this small-animal model. Finally, we adapted endothelial seeding in a modular approach to create scalable and vascularized tissue constructs. ECs were seeded onto submillimeter-sized collagen gel cylindrical modules that contained a second cell (e.g., hepatoma G2, smooth muscle cells, or [most relevant here], cardiomyocytes) [115]. With a view to creating uniform, scalable, and vascularized constructs, these modules were packed into a larger tube, formed into a sheet, or implanted directly with interconnected channels lined with ECs resulting from the random assembly of the modules. These channels connected with the host vasculature in vivo [116], creating a perfusable chimeric vasculature containing both host and donor cells and with host smooth muscle cell involvement. Embedded cardiomyocytes formed "contractile" structures near the periphery of modules, although the density of such structures was relatively low [115]. Remodeling occurred in vivo (after periinfarct injection or use as a patch), resulting in a well-distributed microvasculature (after 2 or 3 weeks in syngeneic animals), but the distribution of cardiac structures was relatively low.

Tissue vascularization is an enduring challenge in the field of tissue engineering and numerous approaches have been devised to tackle it. Advances in microfabrication techniques provided us with increasing control over the spatial arrangement of cells when creating a 3D functional tissue. Building on this progress, we developed a 3D stamping technique based on conventional soft-lithography, which allowed us to pattern thin sheets of synthetic biodegradable polymers and stack them to form a scaffold with a built-in intricate microchannel network mimicking the native blood vessels. The scaffold (referred to as AngioChip) was subsequently populated with ECs within the microchannels and parenchymal cells in the surrounding matrix (Fig. 61.6A) [117]. The stable synthetic scaffold structure supports extensive tissue remodeling, resulting in a free-contracting cardiac muscle and a metabolically functional liver embedded with an internal perfusable vascular network (Fig. 61.6B). The built-in blood vessels were shown to be mechanically stable and permeable, and even allowed vascular sprouting under the guidance of angiogenic growth factors. Furthermore, we surgically connected the built-in vessel network within the engineered tissue to the host circulation system, establishing blood perfusion immediately upon implantation (Fig. 61.6C). This demonstration shows the feasibility of our bottom-up approach to overcome the lack of tissue survival after implantation caused by the absence of vascular perfusion.

Host Response and Biocompatibility

Questions related to the immune and inflammatory response to tissue constructs are starting to draw attention. The host response to a tissue engineered construct is manifested by the innate and adaptive immune systems, involving both plasma (e.g., complement) and cellular components (e.g., macrophages, T cells, etc.), that are directed against engineered cells and grafts or the materials used in tissue constructs. This potent immune response is most often mediated by major histocompatibility complex mismatches between donor and host tissue in allogeneic transplantations. This response can also be manifested in situations in which autologous cells or tissues are engineered to express therapeutic but foreign factors, or if these autologous cells are placed in tissue constructs that themselves negatively affect immune consequences [118].

Immunosuppressants have enabled the successful transplantation of kidneys, hearts, and other organs. With the advent of tissue engineering, new configurations of tissues and organs (often with an added biomaterial component) are being developed and our understanding of the immune and inflammatory response to these new therapies is being shown to be inadequate. Some xenogeneic cell transplants (mice to rat) survive in situations of cardiac repair despite the species differences [119], although this may be specific to the animal model or to cardiac repair. The longevity of a transplant also depends on the ability of somatic cells to withstand and respond to the stresses of

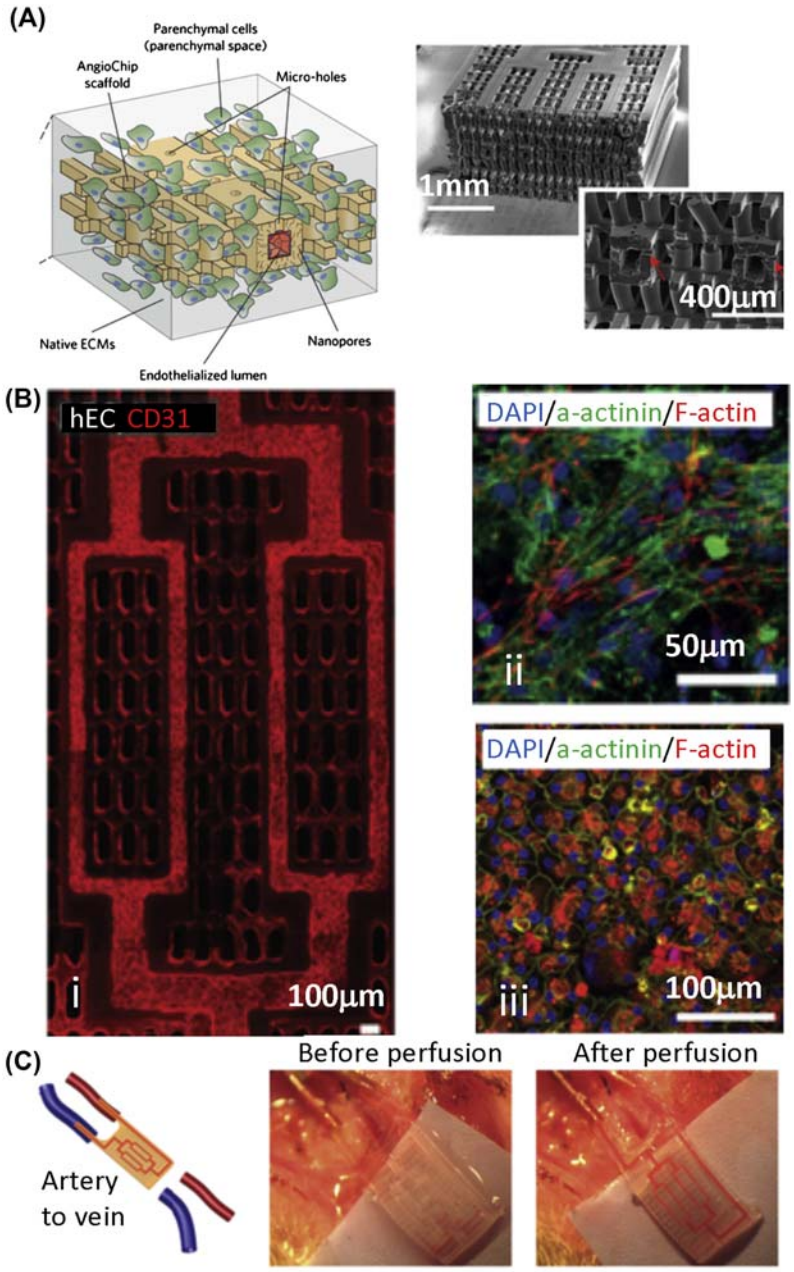

FIGURE 61.6 **Microfabrication of vasculature [117].** (A) Schematic of the AngioChip scaffold illustrating the endothelialized lumen in the parenchymal space (left). Scanning electron micrograph of a multilayered AngioChip scaffold. *Red arrows* illustrate the 20-μm microholes designed to allow perfusion through the AngioChip (right). (B) Immunostaining of the hEC (human endothelial cell) vascular network for (i) CD31 (red), (ii) cardiac tissue stained for sarcomeric α-actinin (*green*), and F-actin (*red*), and (iii) liver tissue stained for E-cadherin (*green*), albumin (*red*), and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (*blue*). (C) Image of the AngioChip in an artery to vein graft in a rat. After the surgical anastomosis, blood perfusion was resumed.

implantation, rejection, and other injuries [120]. The classic "foreign body reaction" to biomaterials is well-known, but the details of the molecular signals (complement regulatory proteins and MMPs) that accompany this phenomenon (in the context of biomaterials) are only beginning to be defined.

A variety of approaches have been undertaken or are under development to generate or improve upon graft acceptance [121]. These approaches include methods to block the innate immune response such as by using drugs or transferring genes to block nuclear factor-κB signaling pathways, for example. Other methods to block the innate response include the use of antibodies to IL-1 or TNF or the use of antiadhesion and antielastase antibodies. We must

understand the mechanism of the host response itself better so that we can design better biomaterials, select or engineer more suitable cells, or devise better strategies to control both innate and adaptive immune responses and enable a functional integration of the new tissue with the host.

IN VIVO STUDIES

In Situ Cardiac Tissue Engineering via Injection of Cells in Hydrogels

Hydrogels have gained much attention as vehicles for the delivery of reparative cells into the myocardium owing to their injectability and ability to control cross-linking chemistry. For a hydrogel for use in myocardial regeneration, it needs to be: (1) biocompatible; (2) biodegradable; (3) injectable, so that it can be applied with a syringe in a minimally invasive manner; and (4) mechanically stable enough to withstand the beating environment of the heart. In addition, a biomaterial that can promote the attachment and survival of cells and localize them at the infarction site would address these limitations of poor cell retention and survival.

Early studies relied on cell injection using natural hydrogels such as Matrigel [122,123] or fibrin [124–126] and reported structural stabilization, reduced infarct size, and improved vascularization upon injection of undifferentiated ESCs [122,123] or bone marrow cells [124–126]. Alginate alone was demonstrated to reduce pathological remodeling and improve function [127]. A synthetic, self-assembling peptide hydrogel (AcN-RARADADARARADADA-CNH) was also used, forming a nanofibrous structure upon injection into the myocardium that promoted recruitment of endogenous ECs and supported survival of injected cardiomyocyte (CM) [128]. Insulin-like growth factor-1 bound to the self-assembling peptide was demonstrated to improve grafting and survival of CM injected into MI [129]. Laflamme and Murry demonstrated that targeting of multiple pathways, which was related to cell survival by encapsulating a number of biomolecules in Matrigel, significantly increased the survival and grafting of the hESC-derived CM injected into infarcted rat hearts [130]. They carried on studies using the same hydrogel to deliver hESC-derived cardiomyocytes in guinea pig [131] and nonhuman primates [132], demonstrating that the cells delivered with the hydrogel could indeed integrate, upon a remodeling phase that included the presence of significant ventricular arrhythmias.

Zhang et al. studied the effect of injecting CM in a mixture of collagen type I and Matrigel [133], the material used by Zimmerman et al. to create engineered heart tissue [134], in MI-induced rats. An additional problem with using ECM proteins in this setting may be the immune response exhibited by rats to mouse proteins (i.e., Matrigel is a basement membrane derived from mouse sarcoma). Positive connexin-43 staining was found in cells in the biomaterial, and the biomaterial was also seen to improve the thickness and function of the heart. The main drawback is that the material takes 1 h to gel, which could allow for significant cell loss, although no cell retention studies were conducted in these experiments.

We modified chitosan with the peptide QHREDGS derived from angiopoietin-1, the peptide sequence implicated in the survival response of muscle cells cultivated in the presence of this growth factor [135]. The chitosan was rendered photocross-linkable by modification with azidiobenzoic acid (Az-chitosan) [136]. Neonatal rat heart cells cultivated on cross-linked films of Az-chitosan–QHREDGS attached, elongated, and remained viable whereas they exhibited lower attachment levels and decreased in viability when cultivated on the chitosan substrates modified with the scrambled peptide sequence [137]. Interestingly, cells on Az-chitosan–QHREDGS were capable of resisting Taxol-induced apoptosis, whereas those on Az-chitosan–Arg-Gly-Asp-Ser were not [137]. We also demonstrated that QHREDGS peptide was able to attenuate pathological remodeling and improve cardiac function upon MI in a rat model, acting largely by promoting the survival of spared cardiomyocytes [138].

Other studies collectively indicated that an injection of hydrogel alone, without the reparative cells, may attenuate pathological remodeling upon MI [127,139–141]. For example, injection of alginate or collagen alone improved LV function and reduced cardiac remodeling after infarction [127,142]. Two alginate biopolymers were modified to assess therapeutic potential in rat MI models. Alginate modified with 0.025% v/v polypyrrole, a conductive polymer, injected into the infarct zone showed improved arteriogenesis at 5 weeks posttreatment and significantly enhanced infiltration of myofibroblasts into the infarct area compared with saline and alginate-only controls [143]. Also, Arg-Gly-Asp–conjugated alginate and alginate alone injected into the infarct zone showed improved LV function and increased arteriole density 5 weeks after injection compared with bovine serum albumin in phosphate-buffered saline control [144]. Results from both studies again show the potential for non–cell based therapies to treat chronic heart failure.

An interesting example is the injection of a hydrogel based on decellularized porcine ventricular myocardium, which resulted in improvements in local and global cardiac function [145,146] and thus the initiation of Phase I clinical trials with this hydrogel.

It is suspected that by changing the ventricular geometry and mechanics, hydrogels reduce the elevated local wall stresses that have been implicated in pathological remodeling [147]. Finite element modeling of wall stresses indicated that upon injection of the material of elastic modulus 10−20 kPa in the infarct, the relationship between ejection fraction and the stroke volume/end-diastolic volume was improved. In addition, injections of the material in the border zone decreased end-systolic fiber stress proportionally to the volume and the stiffness of the injected material.

We believe that properly tuning the mechanical properties of a hydrogel and providing bioactive molecules may offer new cell-free treatment options for MI. The death of CM by necrosis and apoptosis peaks at 6 h upon acute MI [148]. However, the persistent and progressive loss of CM in neighboring areas of the infarct continues up to 60 days after the onset of MI. During this process, up to 35% of cells at the borders of subacute and old infarcts may become apoptotic [149], compared with only 1% in the remote regions of myocardium [150]. Studies in rats and dogs demonstrated that CM loss by apoptosis persists for 1−4 months upon MI, which correlates with the progressive worsening of the pump function. Thus, developing hydrogels that specifically prevent apoptosis of the heart cells (e.g., QHREDGS peptide-modified chitosan) may result in new treatment options in the future, in which hydrogel injected alone in the border zone, without reparative cells, would act to mechanically stabilize the ventricle and prevent further apoptosis of cardiomyocytes.

For example, it was shown that EC-induced cardiomyocyte protection after infarction occurs through PDGF-BB signaling. Thus, binding PDGF-BB to the self-assembling nanofibers of RAD16-II (a peptide consisting of alternating RAD domains, AcN-RARADADARARADADA-CNH_2) hydrogel was evaluated as a potential therapeutic option. Sustained, targeted release of this signaling molecule to host myocardium was observed up to 14 days after injection. Injection of nanofibers with PDGF-BB at the site of infarct in rats decreased CM death and preserved systolic function after MI and showed (separately) a decrease in infarct size after ischemia−reperfusion [151].

The relative contribution of cells versus the injected biomaterial to the attenuation of pathological remodeling also needs to be assessed and the mechanism by which various cells induce functional improvements needs to be elucidated. Although with the injection of contractile cardiomyocytes the expectation is that cells will functionally couple to the host myocardium and contribute to contractile function, the same is not possible for noncardiomyocytes. The exact mechanism by which nonmyocytes impart improvement in function and attenuation of pathological remodeling is still under debate, but some researchers suggest that the transplantation of healthy cells results in the release of growth factors and other molecular signals, i.e., the paracrine effect. These help with angiogenesis, cell survival, and recruitment of progenitors. A possible drawback of using the biomaterial is that the scaffold or hydrogel may also take up space that would prevent a high tissue density until the material degrades.

Implantation of Cardiac Patches

Significant progress has been made in constructing in vitro cultivation systems and biomaterial scaffolds, but fewer studies have focused on implanting cell-based cardiac patches onto viable or injured myocardium. In a pioneering study, Li and colleagues [18] implanted a construct based on neonatal rat cardiomyocytes and collagen sponges onto the surface of the cryoinjured myocardium of Lewis rats. The grafts were implanted 3 weeks after infarction. After 5 weeks in vivo, cells survived supported by blood vessel ingrowth and integrated with the surrounding tissue. However, the graft did not improve LV function.

Attenuation of pathological remodeling (i.e., prevention of ventricle dilatation and maintenance of contractile function) was observed in a study by Leor et al. [20], in which cardiac constructs based on neonatal rat cardiomyocytes and porous alginate scaffolds were implanted onto myocardium of Sprague−Dawley rats that underwent permanent main coronary artery occlusion. The grafts were implanted 7 days after MI. After 9 weeks of implantation, the grafts demonstrated integration with host myocardium at the anchorage sites as well as inflammatory infiltrates and the presence of fibrous collagen.

Zimmerman et al. [15] placed cardiac tissue rings cultivated in the presence of mechanical stimulation onto uninjured hearts of Fisher 344 rats for 14 days. They noticed that although both cells and collagen were isolated from Fisher rats, immunosuppression was required to maintain of heart tissue upon implantation. In the absence of immunosuppression, even in the syngeneic approach, cardiac constructs completely degraded after only 2 weeks in vivo. It is unknown what caused the response; it is possible that it was the remainder of serum or chick extract.

Regardless, the finding has significant implications in the potential implantation of cardiac patches in clinical settings.

To decrease the potential immunogenicity of their engineered tissue, Zimmerman and colleagues discarded all xenogenic components from their culture [152]. This included cultivating the engineered heart tissues in serum-free and Matrigel-free conditions. Mixed heart cell populations rather than cardiomyocyte-rich populations were used and the culture medium was supplemented with triiodothyronine and insulin [152]. Other studies also established the need for nonimmunogenic media. Schwarzkopf et al. used autospecies sera, in this case rat, to culture rat cardiomyocytes [153]. The metabolic activity of cells was significantly higher than for the cells cultivated in conventional culture medium with fetal bovine serum.

Zimmermann et al. demonstrated integration and electrical coupling of a complex multiloop graft to native myocardium in rats with left anterior descending coronary artery ligation (Fig. 61.7A and B). Functional improvement was demonstrated not to be merely the result of scar stabilization or paracrine effects [134]. Functional integration of cardiac cell sheets to the heat-injured myocardium was also demonstrated [154].

In a study performed by Weinberger et al., human engineered heart tissue strips composed of fibrinogen and Matrigel with suspended human iPSC-derived cardiomyocytes were created [155]. The engineered strips, which were intended to limit the number of cells that were washed out after a cell injection, were sutured onto the hearts of cryoinjured guinea pigs. Hearts of the guinea pigs treated with the engineered strips had an improved LV function compared with animals that received a cell free patch, and the graft was found to be electrically coupled to the host myocardium. However, 28 days after implantation, cells were found in the spleen and lungs of some of the guinea pigs.

Although cardiac patches still require further development and regulatory approval, there are numerous in vivo studies improving their cell retention and proving their effectiveness. A cardiac patch developed by Zhang and colleagues was transplanted onto an MI porcine model to test whether including multiple cell types and growth factors would aid in cell retention [156]. The patch was composed of fibrin loaded with insulin growth factor and was encapsulated with a combination of human iPSC-derived cardiomyocytes, ECs, and smooth muscle cells. After transplantation, the group saw that the cell-laden patch increased ejection fraction and the ventricular wall thickness while it decreased the infarct size, which suggested that incorporating a growth factor into the cardiac patch and multiple cell types may be required for the success of such a therapeutic approach (Fig. 61.7C and D).

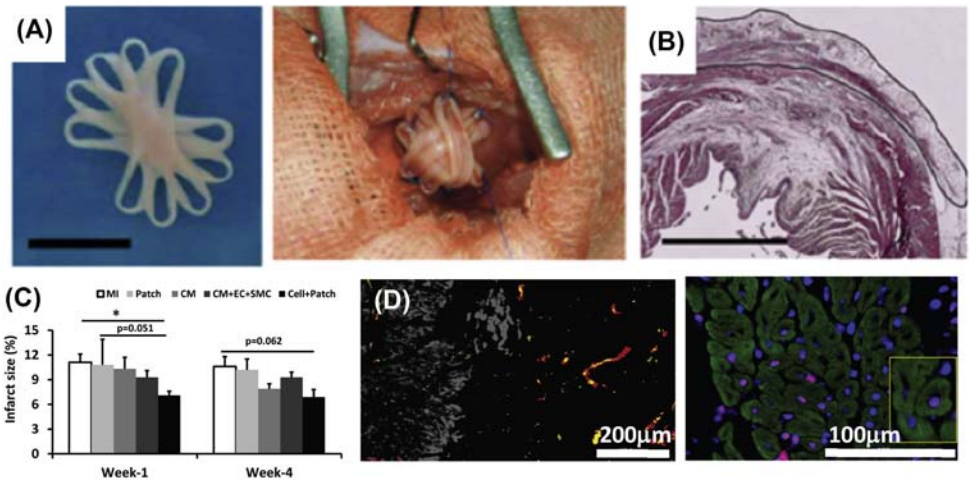

FIGURE 61.7 **In vivo integration of engineered cardiac patches.** (A) A multilooped, mechanically stimulated cardiac construct based on neonatal rat cardiomyocytes and collagen hydrogel was used to repair myocardial infarction (MI) in the rat heart [134]. (B) Histology of the multilooped patch showing integration with the surrounding myocardium [134]. (C) A cardiac patch implanted on an MI porcine model was shown to decrease the infarct size compared with animals not treated with a patch [156]. (D) Immunostaining of the implanted patch shows increased vascular density at the border zone. Image on the left is stained for CD31 (*green*), smooth muscle actin (SMA) (*red*), and cardiac troponin T (cTnT) (*white*). In addition, less apoptotic cells were found at the border zone. Image on the right is stained for apoptotic cells (terminal deoxynucleotidyl transferase dUTP nick end labeling assay) (*red*), cardiac troponin I (*green*), and 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) (*blue*) [156].

The first human clinical trial using a patch-based therapeutic was performed by Larghero and colleagues. In this case report, hESC-derived cardiac progenitors were embedded in a fibrin glue scaffold [157]. The pericardium over the infarcted area was retracted, creating a pocket with which the patch was placed. At 3 months after surgery, the patient's ejection fraction had improved from 26% to 36% and a reduction in end-diastolic and systolic volumes was noted. Despite the increase in symptomatic performance, this trial was conducted only on a single patient; therefore, it is difficult to draw conclusions. However, this trial shows the potential for such a treatment and future clinical trials.

In addition to engineering the patches of myocardium, Zimmermann and colleagues designed the first biological assist device [158]. The authors mechanically stimulated a hollow-spherical construct consisting of collagen I and neonatal rat cardiomyocytes until a beating pouch-like structure was created. The pouch was then placed over uninjured rat hearts in such a manner that the right and left ventricles were covered. Fourteen days after implantation, the pouch covered the epicardial surface of the heart and exhibited blood vessel ingrowth.

Roche et al. created an alternative ventricular assist device that uses soft robotics to pump the heart manually. The sleeve consists of actuators aligned helically to mimic the twisting motion of the beating heart. It was tested in vivo on a pig model with acute heart failure and the researchers observed the reestablishment of cardiac output. The group hopes to see the technology used in the clinic as a device to extend the life of a patient waiting for transplant [159].

Badylak et al. implanted ECM derived from porcine urinary bladder into a surgically created 2-cm [2] defect in the LV free wall of dogs. Eight weeks after implantation, the ECM patches showed higher regional systolic contraction compared with the control group in which a material (Dacron) used for myocardial defects was employed. Histological analysis suggested that cardiomyocytes accounted for about 30% of the remodeled tissue in the ECM scaffolds [160]. In another study, this improvement in the heart function was attributed to an increase in the myocyte content in the ECM patches between weeks 2 and 8. The relationship between the myocyte content and the extent of mechanical function was observed to be linear. There was also some evidence (a decrease in cardiomyocyte diameter and an increase in the overall area occupied by cardiomyocytes over time) suggesting the possibility of cardiomyocyte proliferation in the patches [161].

Limitations related to the source of autologous cardiomyocytes motivated studies that used nonmyocyte-based patches for MI repair. Smooth muscle cells seeded with poly (ε-caprolactone-*co*-L-lactide) sponge reinforced with poly-L-lactide fabric were used in a modified endoventricular circular patch plasty procedure (Dor procedure). Cell-seeded grafts resulted in improved LV function (as assessed by echocardiography) compared with cell-free controls [162]. A patch made of dermal FBs seeded onto knitted Vicryl mesh (Dermagraft) was used in an attempt to increase angiogenesis upon MI. When placed over the infarcted regions on the hearts of SCID mice, the grafts improved microvessel density within the damaged myocardium [163].

There appears to be a consensus regarding the requirement for multiple cell types, specifically FBs and ECs, in addition to cardiomyocytes for successful cardiac tissue graft survival and vascularization in vivo. In one approach, omentum was used to prevascularize cardiac patches based on neonatal rat cardiomyocytes and alginate scaffolds modified with angiogenic factors. After excision and implantation into the infarcted rat myocardium, the vascularized cardiac patch showed structural and electrical integration into host myocardium and attenuated pathological remodeling of the ventricle significantly better than did the in vitro cultivated patch alone [164].

In another strategy, a simultaneous triculture scaffold-free approach was used to generate beating cardiac patches based on hESC. Upon implantation into the hind-limb muscle of nude rats, these patches, which were composed only of enriched cardiomyocytes, did not survive to form significant grafts. However, patches containing ECs (either HUVECs or hESC-derived ECs) and FBs in addition to cardiomyocytes persisted in the (noninfarcted) rat heart and resulted in 10-fold larger cell grafts compared with cardiomyocyte-only patches. The preformed human microvessels also anastomosed with the rat host coronary circulation and delivered blood to the grafts [165].

These studies demonstrated the feasibility of cardiac patch implantation, but further studies are necessary to estimate the effect of culture conditions and scaffold type on the in vivo outcome. Although significant progress has been made in the area of biomaterials and bioreactors, it is unknown which cultivation conditions and what biomaterial will best preserve contractile function and prevent pathological remodeling upon implantation. Thus, studies that investigate this systematically and correlate in vitro parameters (e.g., force of contraction) to in vivo outcomes (e.g., fractional shortening) are required. The host response to the patch and the nature of the immunologic situation further complicate these studies.

Delivery Route for Cells and Patches

It remains to be determined how and where in the heart the cells and patches should be applied for optimal therapeutic benefit. The ventricular wall is composed of the *epicardium*, the outer epithelial lining of the muscle; the *myocardium*, composed of working CMs and support cells such as **FBs**; and the *endocardium*, consisting of a specialized endothelial lining that is in contact with blood filling the ventricle. Most cell therapy approaches focus on the direct injection of a cell suspension into the myocardium using either catheters or open chest surgery [166–168]. These approaches are inherently limited by the small volume of free space available for cell engraftment. The cell density in the myocardium is extremely high (10^8 cells/cm^3 [169]); upon injury such as MI the dead cells are replaced by a dense collagenous scar, leaving no space for injected cells to engraft. The injected cells are rapidly washed out or ejected, resulting in a maximal engraftment efficiency of 10% [20,130,170,171], which necessitates the injection of as many as 1 billion CMs for sizable grafts in monkeys [132].

The injection of isolated cells also offers little opportunity to mature or modify the electrophysiological properties of the graft. The immaturity of the injected cells was postulated to have caused malignant arrhythmias in monkeys that received hESC-derived CMs [132]. Thus, cardiac patches may offer a unique solution to the challenges of cell delivery, engraftment, and graft function. The size, shape, and functional properties of cardiac patches can be tailored through in vitro cultivation with electrical field stimulation [54,73] or mechanical stimulation, whereas their application on the ventricular surface could provide sufficient space for implant engraftment to a clinically relevant size. In fact, in a clinical trial, hESC-derived cardiac progenitors were applied during open heart surgery onto the *epicardial surface* of the heart using a fibrin hydrogel and a pericardial patch over the hydrogel [157]. The epicardium is generally considered a safe location for patch deployment, owing to the high risk for embolization and thrombosis associated with patch application inside the ventricle on the endocardial surface of the ventricular wall.

SUMMARY

Functional viable cardiac patches have been engineered based on neonatal rat cardiomyocytes and hESC- and iPSC-derived cardiomyocytes. Various biomaterials have been tested for this purpose and in vitro culture systems have been developed that enhance cardiac construct differentiation (mechanical and electrical stimulation), improve cardiomyocyte survival at high density (medium perfusion), and enhance maturation of cardiomyocytes derived from human pluripotent stem cells, paving the way to engineering autologous cardiac patches of clinically relevant size.

The field of microphysiological organ systems and organ-on-a-chip engineering has emerged and matured to the level required to use these miniaturized 3D cardiac tissues in drug discovery, safety testing, and disease modeling, relying on human cardiomyocytes derived from iPSCs.

Because the in vivo studies conducted thus far used different cell sources, biomaterials, animal models, delivery times after infarction, and experimental time frames, direct comparison cannot be made between methods. Although all reported studies have shown some form of improvement, complete myocardial regeneration has not been achieved. Perhaps a valid question to be answered in the future is: What is the required level of myocardial regeneration in terms of survival and attenuation of symptoms? Complete regeneration is an ambitious goal that may not be required. Future studies must also increase their time frames to assess the long-term effects of these treatments better.

Although the completely artificial heart will remain a dream, the near future may bring a clinically relevant autologous cardiac patch as evidenced by rapid progress in engineering cardiac patches based on stem cell–derived cardiomyocytes. Work on recellularizing decellularized hearts may represent the first step toward "the heart in a box" envisioned several decades ago.

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Further Reading

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CHAPTER

62

Bioengineering of Liver Tissue

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INTRODUCTION

The liver is the largest internal gland in the body and one of the most critical organs in metabolic homeostasis because it is responsible for many essential metabolic exocrine and endocrine functions. Among them, it is responsible for the detoxification and elimination of a variety of substances, regulation of glucose levels, maintenance of blood homeostasis, and production of many products including lipids, proteins, vitamins, and carbohydrates. In addition to this, the liver possesses a unique regenerative capacity; it is able to regenerate most of its function after losing up to three-quarters of its mass as a result of partial hepatectomy or toxic injury. However, in severely ill patients with an end-stage liver disease, this regeneration capacity is severely limited and liver transplantation (LT) becomes the only lifesaving intervention. This also applies to patients with decompensated cirrhosis, hepatocellular carcinoma (HCC), inborn errors of metabolism, and fulminant hepatic failure. Moreover, LT remains the only available cure for a wide range of liver diseases.

Nearly a half century has passed since Dr. Starzl [1] performed the first liver transplant. However, it was not until 1967 when short-term success was achieved with 1-year survival after transplantation [2]. Since then, the success and the survival rates of LT have increased owing to improved immunosuppressive regimens, surgical techniques, and donor–recipient pairing [3]. However, today, the number of patients waiting for a liver transplant far exceeds the number of transplants performed. Although this gap was reduced with the increased use of marginal organs such as steatotic livers, employment of hepatitis C virus (HCV)-positive donor organs for HCV-positive recipients, and donation after cardiac death, the number of patients waiting for a liver transplant continues to exceed the number of available livers.

According to reported by the Organ Procurement and Transplantation Network in 2014, 6729 liver transplants were performed in adults whereas around 14,600 people were on the waiting list to receive a donor's liver. Although 6729 transplants were performed, 1821 patients died while waiting for a donor and 1290 patients were removed from the list because they were too sick for transplantation during that year [4]. Despite attempts to reduce the waiting time, two-thirds of all patients will never receive one.

Liver cell transplantation has also been considered a possible treatment for patients with life-threatening liver diseases. In this case, a preparation of isolated cells is injected directly into the liver via a portal vein or into the spleen. Engraftment of these cells and their therapeutic potential to correct many metabolic deficiencies has also been demonstrated by several studies [5]. In 1992, a group of scientists from Michigan University performed the first successful hepatocyte transplantation into a French-Canadian woman with familial hypercholesterolemia [6]. Afterward, other successful cases followed, but not all patients had noticeable progress [7], which was also observed with other diseases [8,9]. Although the long-term safety of this technique has been confirmed [10,11], the success

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of this procedure has not yet been enough to evade the need for whole-organ transplantation. One possible explanation for these results could be the small number of hepatocytes that engraft in the recipient owing to the poor quality and quantity of the cells used [12]. Hence, it is accurate to say that as a result of hepatocytes, the shortage of human liver donors is the limiting factor in the development of this therapeutic approach. Other sources such as hematopoietic stem cells [13], bone marrow–derived mesenchymal stem cells [14,15], and fetal liver progenitors [16,17] have also been used (Fig. 62.1).

In addition to cellular therapies, other experimental approaches are in development with high therapeutic potential. These novel approaches aim to stimulate the diseased liver to regenerate itself or substitute it with a bioengineered liver capable of performing all native liver functions [18]. In this regard, and with the available technology, liver bioengineering comprises the creation and use of a supporting scaffold and large numbers of hepatic and vascular cells to generate functional tissue. Considerable effort has been made in trying to develop biomaterials capable of mimicking the liver extracellular matrix (ECM) to replicate the benefits offered by this structure regarding cell adhesion, growth and viability, and maintenance of the differentiation state and metabolic functions. Despite this, in the first studies performed with synthetic materials, the maintenance of the vascular network was not successfully achieved, resulting in low hepatocyte survival [19]. Some of these obstacles were solved by administering growth factors to promote angiogenesis and increase cell viability [20,21], but also by developing advanced bioreactors to improve hepatocyte seeding [22]. However, the absence of specific hepatic signals for cell growth and differentiation proved to be another critical limitation, probably owing to the lack of a real three-dimensional (3D) environment in which hepatocytes could find or create their native niche. Several research groups have addressed this issue by employing tissue decellularization to prepare bioscaffolds devoid of all cellular material from a harvested tissue or organ. In 2009, Baptista et al. successfully repopulated decellularized rat and ferret livers with

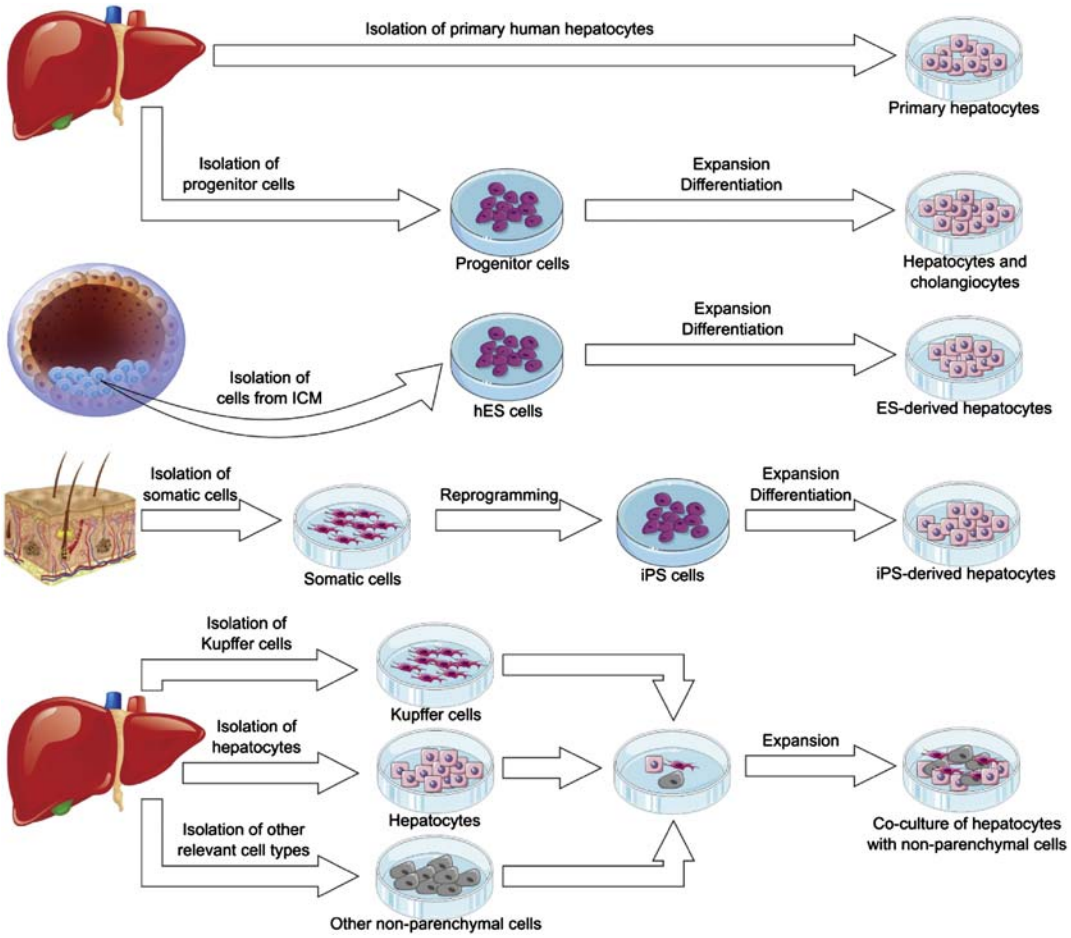

FIGURE 62.1 Available strategies for the isolation and generation of hepatic cells and tissues. *ES*, embryonic stem cells; *hES*, human embryonic stem cells; *ICM*, inner cell mass; *iPS*, induced pluripotent stem cells.

human fetal liver and endothelial cells [23,24]. Immediately afterward, Uygun's group achieved the decellularization of rat livers, which were then repopulated with rat primary hepatocytes and anastomosed ex vivo with a rat for up to 8 h [25]. It is still difficult to know the real value of this technology in clinical applications, but the potential to change transplantation medicine and patients' lives is evident.

Hence, in this chapter, we will describe some of these novel regenerative medicine approaches and the potential applications for these tissue-engineered liver constructs as biologic surrogates for a large variety of biomedical and pharmaceutical applications.

HEPATIC TISSUE ENGINEERING

It is widely accepted today that 3D cultures mimic in vivo natural conditions much better than do traditional 2D cultures, in which cells are grown on a flat surface in Petri dishes. The reason for this is that 3D cultures more closely reflect in vivo cell behavior, providing a physical and mechanical environment that promotes cell proliferation, differentiation, and expression of specific growth factors and other molecules. Hepatic tissue engineering has been able to create bioengineered grafts with the medical purpose of organ replacement, or restoration, which recreate physiological 3D microenvironments that more accurately mimic the in vivo environment. These promising techniques rely on the combination of different disciplines such as engineering, biomaterials, cells, and growth factors.

Starting with the biomaterials used, these can be made of either natural or artificial components capable of supporting 3D cellular growth. Some examples of their applications in tissue engineering are described subsequently.

Chitosan

Chitosan is a natural polysaccharide derived from chitin, a structural polymer of arthropod exoskeletons [26], which has been widely used in tissue engineering because of the structural similarity with glycosaminoglycans, another component of liver ECM [27,28]. Fan et al. [28] combined chitosan with galactosylated hyaluronic acid, making porous 3D sponges that induced hepatocyte aggregation that displayed liver-specific metabolic activities.

Gong et al. used chitosan−gelatin liver scaffolds with a hierarchical predesigned channel network inside, providing a porous structure. They compared this fabricated scaffold with conventional porous ones, obtaining higher proliferation rates (62%) of HepG2 [29]. Jiankang et al. [30] also designed a model of a porous scaffold made of chitosan and gelatin with internal structures such as portal vein, hepatic chambers, well-organized fluidic channels, and central vein, demonstrating improved hepatocyte aggregation and liver-specific functions. In another experiment, Zang et al. [31] determined that 1% of genipin cross-linked with chitosan−gelatin scaffolds present better results in HepG2 performance compared with glutaraldehyde, and r1-(3-dimethylaminopropyl) 23-ethyl-carbodiimide hydrochloride cross-linked scaffolds.

Galactosylated chitosan (GC) is also commonly used, combined with hyaluronic acid, an anionic glycosaminoglycan widely present in mammal ECM, to create porous sponges in which hepatocytes and endothelial cells were cultured [32]. This hybrid scaffold could represent a cell storage and delivery vehicle for a bioengineered liver tissue. Chung et al. [33] combined GC with alginate, creating a highly porous 3D sponge for hepatocyte anchorage. They demonstrated better spheroid formation and higher cell viability compared with scaffolds made only of alginate. Chitrangi et al. compared three different scaffold constructions for the differentiation of human umbilical cord-derived mesenchymal stem cells into hepatocyte-like cells: dextran−gelatin, chitosan−hyaluronic acid, and gelatin-vinyl acetate. They reported better results in hepatocyte differentiation when using those scaffolds made of gelatin, probably owing to its presence in natural ECM composition [34].

Collagens

Type I collagen, a crucial component of mammal ECM, is one of the most used materials in tissue engineering, especially for hepatocyte culture and primary hepatocyte transplantation [35]. Risbud et al. published several articles in which they compared the use of hydrogel scaffolds coated with collagen or with chitosan, demonstrating better human umbilical vein endothelial cell attachment and growth in the first ones. They also suggested that collagen hydrogel-coated textile scaffolds represented an excellent system in which hepatocytes and nonparenchymal cells could be cocultured in parallel [36,37].

Matrigel consists of a complex mixture of ECM proteins (collagen IV), proteoglycans (heparan sulfate), and growth factors (epidermal growth factor and transforming growth factor-β) secreted by Engelbrecht−Holm−Swarm mouse sarcoma cells that resemble the ECM of many tissues. Although the main inconvenience of Matrigel is that it is not a well-defined culture condition, many authors use this compound in vitro. For example, Fan et al. combined Matrigel with agarose to construct a hybrid hydrogel to obtain 3D constructs for tissue engineering [38]. Yarmush et al. also developed 2D culture systems that rely on using mixed collagen I and Matrigel sandwiches. These systems showed that primary hepatocyte function was maintained for several weeks without a noticeable loss of function [39].

Alginate

Alginate, a hydrophilic porous polysaccharide matrix that allows cell growth and differentiation, is also commonly used. Shteyer et al. [40] suggested that alginate scaffolds improve animal survival after an acute hepatic failure owing to an 87% partial hepatectomy in mice, achieving in vivo hepatocellular function of primary hepatocytes. They compared these results with collagen scaffolds and obtained inferior results. Lin et al. used alginate scaffolds for bone marrow−derived mesenchymal stem cell differentiation into hepatocyte-like cells [41]. Tai et al. [42] suggested in vitro differentiation of human mesenchymal stem cells into hepatocyte-like cells using an Arg−Gly−Asp−modified chitosan−alginate scaffold. This construct was implanted in rats with a 70% partial hepatectomy and used for the in vivo delivery of liver-like cells.

Many other related studies describe the power of alginate matrices as scaffolds in liver tissue engineering. They enhance hepatocyte viability, morphology, and function, maintaining hepatocellular performance [43]′ [44].

Polyglycolic Acid, Polylactic-*co*-glycolic Acid, and Polycaprolactone

Synthetic materials have also been used for this purpose, such as polylactic-*co*-glycolic acid (PLGA) or polyglycolic acid (PGA). Lees et al. [45] generated 3D scaffolds made of PLGA coated with laminin in which they seeded human embryonic stem cells. These scaffolds were then transplanted into the liver lobules of mice. The researchers observed the formation of teratomas that produced typical proteins of hepatic lineages and also from pancreatic and neuronal lineages. Furthermore, they found the assembly of an ECM rich in laminin, collagen IV, and collagen I, and positive CD34 endothelial progenitors, which represented vasculature formation.

Barralet et al. [46] developed a PGA fiber mesh scaffold stabilized with polycaprolactone to study the behavior of human biliary epithelial cells. They compared this technique with alternative models as collagen I sandwiches or Matrigel, showing spheroid aggregation, long-term proliferation (6 months), and phenotypic stability, which would represent a good strategy for bile duct tissue engineering. Other studies [22,47] achieved hepatic tissue generation with primary hepatocyte seeding in this PGA scaffolds, with the capacity of albumin and urea secretion.

Decellularized Extracellular Matrix

Decellularization techniques represent a popular technique for generating naturally derived scaffolds. In this process, whole organs are perfused with detergent solutions to remove the cellular contents, preserving an intact ECM and vascular tree. The main advantage of this technique is that there is no need to create an ECM in vitro because the ultrastructure and composition of the native ECM are preserved. In the same way, the vascular network and bile duct system are also well-preserved after liver decellularization [23,24,48].

These scaffolds are then recellularized with different cell types and numbers using different seeding conditions, with the objective of creating functional bioengineered organs able to be transplanted into recipient animals and, in the long-term, into patients with end-stage liver failure.

Human, pig, and rat livers are the scaffolds mainly used for liver tissue engineering, but there are several studies in which spleen was used (Fig. 62.2) [49].

Regarding the decellularization process, there are differences reported by several authors. The most commonly used reagents are sodium dodecyl sulfate [50] and Triton X-100 [23,51,52]. Some other examples are enzymes, proteases, ethylenediaminetetraacetic acid, or deoxycholic acid. There are also multiple concentrations and combinations of these reagents [53,54], several methods of detergent perfusion exist, as reported by several researchers. Some use the portal vein [50], others the portal vein and hepatic artery [23,51,55], and others the inferior vena cava [56]. Cell seeding methods are also variable [53,54]; the portal vein and hepatic vein are the most commonly used.

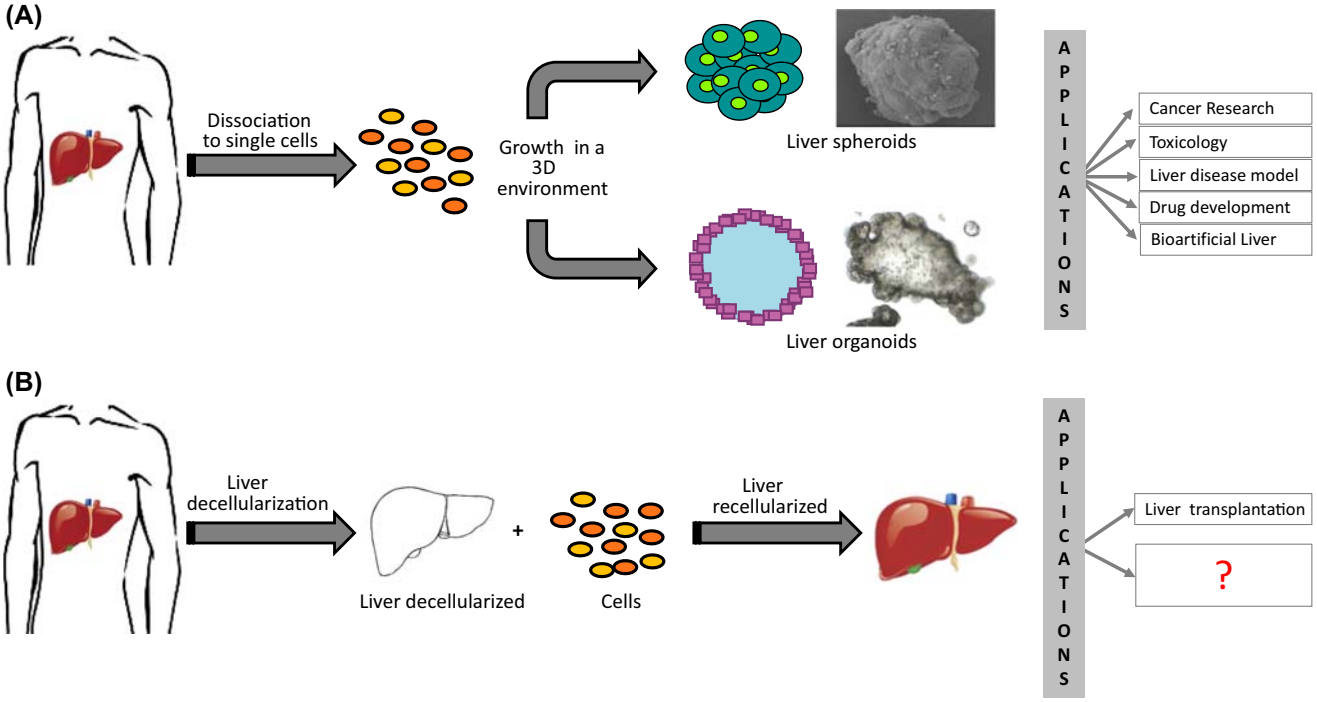

FIGURE 62.2 **Biomedical applications of liver bioengineered tissues**. (A) The resulting single cells isolated from the digestion of human hepatic tissue samples can be seeded in a three-dimensional (3D) environment, originating spheroids and organoids with multiple applications. (B) Cadaveric donor livers can be decellularized and afterward recellularized using the patient's own cells, with the ultimate goal of obtaining a functional liver for transplantation.

Regarding cell types, primary hepatic cells, both parenchymal (hepatocytes) and nonparenchymal cells (sinusoidal endothelial cells, stellate cells, and Kupffer cells), have been used for liver tissue engineering, as well as pluripotent stem cells, fetal progenitor cells, and immortalized cell lines.

Cell number is also a significant variable to take into account [48,57]. To mimic better what is observed in vivo, it is essential to recreate the hepatic spatial distribution (e.g., 80% of the liver parenchyma is composed of hepatocytes). However, there are issues regarding this: not all the cells that comprise the liver structure are easily isolated and maintained in vitro (e.g., hepatocytes do not efficiently expand in vitro, and liver sinusoidal endothelial cells are difficult to obtain) [57]. Furthermore, high cell seeding densities usually provoke cell aggregation, blocking the vascular network perfusion. On the other hand, low cell seeding densities will limit cell−cell interactions, limiting cell growth, affecting both cell viability and survival in the scaffold.

Taking this into account, different recellularization methods have been developed [48,57,58]. Soto-Gutierrez et al. [48] compared three of them regarding better hepatocyte survival and function: direct parenchymal injection, continuous perfusion, and multistep infusion. The first consists of direct cell injection into different lobes of the liver scaffold. In the second technique, the total amount of cells that are going to be seeded are suspended in the culture media and perfused at a determined flow rate. Finally, the cells are added to the culture media in multiple steps, with time intervals between them, and perfused at a determined flow rate. They determined that the best results were using the multistep perfusion system, which not only allowed hepatocyte engraftment around 90%, but also were able to produce albumin and metabolize ammonia and cytochrome P450 (CYP)1A/2 activity for 7 days in vitro.

The flow seeding conditions are also critical. Low flows should be used to allow cell attachment and survival in the scaffold surface. Moreover, every type of cell has its particular cell attachment conditions, with the need to find a particular speed that allows for the most significant cell engraftment as possible, and the delivery into the native spatial location inside a decellularized liver scaffold. Other shortcomings that have to be solved are the long-term cell survival or graft thrombosis after implantation, owing to lack of a functional vascular network in the current generation of bioengineered livers. What can be determined from this is that a lot of relevant issues still need to be addressed in this novel field of liver bioengineering before clinical applications. Although much has been accomplished so far, mimicking in vitro the complexity observed in vivo is still challenging.

Future studies may lead to a better understanding of the production of human-sized transplantable bioengineered livers able to restore diseased organs or replace failed organs.

LIVER SPHEROIDS, ORGANOIDS, AND AGGREGATES: CANCER, BIOARTIFICIAL LIVER, TRANSPLANTATION RESEARCH, AND TOXICOLOGY AND DRUG DEVELOPMENT

Another strategy for the generation of 3D liver tissue constructs is using the hepatocyte capacity to form hepatospheres, which are spontaneous nonadherent spheroid cell aggregations that mimic in vivo cellular environment. The generation of these structures can be achieved by multiple methods such as nonadherent round-bottom wells using methylcellulose-containing medium, rotary and rocked cultures, hanging drops, or 3D matrices [59]. These hepatic structures have revealed higher liver-specific hepatospheres [60,61], which solves the problem of culturing hepatocytes in 2D systems, where dedifferentiation quickly develops. Cocultures with endothelial and other nonparenchymal cells were also developed using this technique (Fig. 62.2A).

Cancer Research

Liver spheroids have emerged as a promising tool in cancer research [62,63]. This 3D culture method has been used as an intermediate model between in vitro and in vivo microenvironments [64]. Studies have demonstrated that liver spheroids preserve a more significant number of liver cells functions better than 2D culture, including albumin and urea production and bile secretion [62,63]. Moreover, the 3D culture conserves cell polarization, resulting in higher concordance with in vivo conditions rather than 2D cultures. This is particularly important in cancer research because 3D cultures better resemble the interplay of the tumor and its microenvironment. This culture configuration allows for the formation of a stable tissue in which cell−cell and cell−matrix interactions are better preserved than in 2D cultures [65]. Hence, liver spheroids more accurately reflect the tumor complexity and heterogeneity and constitute a therapeutically relevant model, displaying pathophysiological gradients of in vivo tumors, such as pH and oxygen gradients, the penetration rate of growth factors, and the distribution of proliferating and necrotic cells [64].

Liver spheroids and aggregates have also been used to evaluate the mechanism of tumor growth, metastasis development, and antitumor therapeutic agents [63,65,66]. Not long ago, unlike other cancers, there was a lack of evidence and data published about the use of liver spheroids in primary liver cancer research. An increase was observed in the number of studies using liver aggregates for in vitro modeling of HCC [67,68]. Song et al. cultivated multicellular tumor spheroids to elucidate the mechanisms of environment-mediated chemoresistance and metastatic transformation in HCC. In that study, the authors created different multicellular spheroids that contained different human HCC lines and stromal cells, including hepatic stellate cells (HSCs). The authors observed that the presence of activated HSCs increased the compactness of spheroids and enhanced resistance to chemotherapeutic agents such as sorafenib and cisplatin compared with other stromal cells. The authors also observed that this increase in drug resistance was related to HSC production of collagen 1A1. On the other hand, the results of the study showed that activation of HSC promoted HCC migration by upregulating matrix metalloproteinase 9. Globally, the authors demonstrated that interaction between HCC cells and HSC induced chemoresistance and gave the tumor a more aggressive and invasive profile; they suggested that HSC could be the target for novel therapeutic strategies [68].

In the same way, Chen et al. used HCC spheroids to screen candidate metastasis-associated genes. The authors built two different HCC spheroids using two different cell lines, one with high-metastatic (MHCC97H) behavior and other with a less aggressive profile (Hep3B). Then the authors evaluated the expression of candidate metastasis-associated genes related to cell adhesion, matrix secretion, and invasion. The results showed an evident change in the gene expression of 123 genes between the two spheroids. In the MHCC97H spheroids, the authors found that the number of upregulated genes related to adhesion molecules mediating cell−matrix interactions and matrix secretion was significantly higher. In contrast, the Hep3B spheroid presented an increased expression of adhesion molecules maintaining cell adhesion. With these data, the authors could identify a specific gene expression pattern that may determine the malignant phenotype of the HCC spheroid [67].

The findings of these studies represent the potential of the liver spheroids in the field of cancer research. 3D culture models offer a more accurate environment of in vivo microarchitecture to study tumor growth and progression.

In the future, the introduction of new biomaterials and biofabrication techniques will also reproduce the interaction between cells and matrices more precisely. For these reasons, the results obtained from studies performed with 3D culture methods are more consistent owing to their similitude with the in vivo reality. A strong implication of liver spheroids in cancer research is their potential in personalized medicine. Future studies in patients with primary liver cancer or liver metastasis may use host-tumor based spheroids to evaluate pharmacologic activity and toxicity.

Bioartificial Liver and Transplantation Research

The liver is a vital organ with complex functions including gluconeogenesis, albumin and urea synthesis, lipid metabolism, drug detoxification, waste removal, and immune and hormonal modulation. Liver diseases are prevalent worldwide; most cases lead to progressive impairment of liver function. The only available treatment for life-threatening conditions such as end-stage liver disease and acute liver failure is LT, but because of donor shortage and an increase in the number of potential LT candidates, alternative measures are needed [69]. Owing to their ability to resemble in vivo structures and preserve organ-specific functions, liver spheroids have also been used in bioartificial livers and transplantation research [62,63]. Liver spheroids have a potential use in this field, directly transplanted or employed in hybrid and bioartificial liver support systems (Fig. 62.2) [70–72].

In 2005, Gan et al. explored the role of a spheroid-based hybrid artificial liver support system (HALSS) for treatment of severe liver failure. In this study, the HALSS consisted of a plasma separator, a hemoabsorbant, and a bioreactor with pig-derived hepatocyte spheroids in its extrafiber space. This system has been used in 10 patients with severe liver failure. The authors observed that after 24 h of spheroid culture, they produced higher levels of albumin and urea in respect to 2D cultures, suggesting better preservation of cell function. The authors also observed that patients treated with HALSS had a better survival rate (30% versus 0%), low levels of total bilirubin and alanine transferase, and improvement in coagulation compared with those in the control group. With these results, the authors concluded that spheroid-based HALSS could be useful in supporting liver function in patients with severe liver failure [71]. A preclinical study by Glorioso et al. evaluated the neuroprotective effect of the spheroid reservoir bioartificial liver (SRBAL) in a porcine model of acute liver failure. In that study, 18 pigs with an acute liver failure caused by a drug overdose were randomized into three groups: standard therapy, standard therapy plus no cell liver support device, and standard therapy plus the SRBAL device. After 90 h of treatment, the authors observed that pigs treated with the SRBAL improved survival compared with standard therapy and no cell device therapy (83%, 0%, and 17%, respectively) and had significant improvement in the rate of ammonia detoxification, peak levels of serum ammonia, and intracranial pressure. The authors also found that hepatocyte spheroids remained highly functional and that survival and device function were directly related to hepatocyte cell dose, treatment duration, and membrane pore size [72]. These studies reflect the potential of the spheroid-based bioartificial liver as a promising treatment for liver failure. Spheroid aggregates offer a readily available source of donor cells and reach the minimal cell dose to obtain a significant clinical effect in animals and humans. Regardless, randomized clinical studies are needed to determine whether these bioartificial support systems are safe and feasible in individuals with acute liver failure, with the aim of expanding its use to clinical practice and avoiding LT.

Other studies have evaluated the effect of direct transplantation of liver spheroids. No controlled trial has yet evaluated the clinical benefit of liver cell therapy in liver diseases in humans, and slightly more than 100 patients have been treated with hepatocyte transplantation [73]. However, the benefit is well-known in animal models. In 2002, Hamazaki et al. demonstrated that intraperitoneal transplantation of microencapsulated multicellular spheroid of rat hepatocytes improved survival in acute liver failure induced by 90% hepatectomy. Nevertheless, data are needed on liver spheroids transplantation in humans [70].

Results from experience with hepatocyte transplantation in humans and with liver spheroids in animals are promising, and cell-based therapies emerged as a “bridge to transplant” for both metabolic liver disease and liver failure. The most significant limiting factor is that sources of hepatocytes for transplant are scarce and tissues that are available come from organs that are unsuitable for LT [74]. To avoid this problem, alternative sources of cells have been evaluated. Pettinato et al. evaluated the differentiation of human induced pluripotent stem cells (hiPSCs) into hepatocyte-like cells through direct Wnt−β-catenin pathway inhibition [75]. The authors developed a new multicellular spheroid-based hepatic differentiation protocol starting from hiPSCs. The resultant spheroids were transplanted into a rat model of acute liver failure. The results showed that transplantation significantly prolonged the mean survival and ameliorated the liver function compared with the control group. These results indicated that this differentiation program is feasible and highly efficient and suggested that the differentiation of stem cells

may provide an alternative source for hepatocyte-like cells with comparable differentiation and function [75]. Once again, these results confirm the power of 3D culture to preserve liver-specific functions throughout time.

Finally, liver spheroid transplantation could also have a role in personalized gene therapy. A study suggested that transplantation of spheroids of cells after genetic modification with nonviral DNA vectors may increase the therapeutic potential of this technique. In their work, Uchida et al. evaluated this hypothesis [76]. The authors transplanted subcutaneously hepatocyte spheroids transfected with erythropoietin DNA in rats. They observed that the spheroid-treated group showed a significantly higher hematopoietic effect than animals transplanted with a cell suspension from monolayer cultures. Moreover, the spheroid system contributed to the preservation of native functions of hepatocytes in the host tissue, confirming once again that 3D culture delays dedifferentiation and loss of functions of these cells [76].

These results introduced a new perspective for liver spheroid transplantation. In the future, genetically modified spheroid transplantation systems may be used to overcome genetic diseases. The actual state of the art suggests that we may soon be able to create and transplant genetically modified spheroids of hepatocyte-like cells derived from the patient's own stem cells, which will represent a revolution in personalized medicine.

Toxicology and Drug Development

The liver is the organ where most drugs are metabolized and transformed into metabolites or active compounds. These substances by-products may become toxic to the liver itself and the rest of the body, a phenomenon known as drug-induced liver injury (DILI). To launch a single drug into the market is a long (12–15 years) and costly ($3–5 billion) process [77]. After an initial screening, lead candidate compounds are characterized in vitro and in vivo for their absorption, distribution, metabolism, excretion, and toxicity properties before proceeding with clinical trials. However, most compounds (>90%) fail during these final stages: 43% of these failures occur owing to a lack of efficacy and 33% to the appearance of adverse effects [78], mainly because of DILI [79]. Hence, drug withdrawals at clinical stages in humans mainly result from the use of inappropriate or inaccurate in vitro and in vivo liver models in the course of drug studies.

On the other hand, the liver is the target organ of some prevalent diseases, such as infectious hepatitis B virus, HCV [80], malaria [81], overnutrition-induced (type 2 diabetes, nonalcoholic fatty liver disease, fibrosis, and cirrhosis) [82–84] or tumoral diseases (HCC represents the sixth most common cancer worldwide) [85].

Considering all of this, liver models' results are necessary for the development of novel drugs, not only for the study of xenobiotics' metabolism and toxicity but also for the development of specific drugs for liver diseases. Hence, more realistic in vitro human liver models are needed that resemble as closely as possible in vivo liver structure, physiology, and the pharmacological response.

Limitations of Current In Vitro Liver Models to Test Drugs

As mentioned before, maintaining liver parenchymal function ex vivo is essential to generating stable systems for efficacy and toxicology drug studies, so fully functional hepatocytes are needed. For that, the 3D relationship of cells within the differential microenvironments of the liver (e.g., periportal versus pericentral), the regional hemodynamic flow patterns, and other physiological factors such as oxygen tension and cytokine profiles have to be simulated in vitro. However, cell-based models that are routinely used in drug testing are simple monoculture systems (typically standard microtiter plate formats) employed under static, nonphysiologic 2D conditions, which make them suboptimal models for drug efficacy and safety testing, unable to mimic or predict more complex mechanisms of action [86].

Hepatocyte viability in suspension decreases significantly after 4 h. Because of that, for years cryopreserved human hepatocytes in monolayer cultures have been the reference standard to test drug metabolism and toxicity [87]. However, cryopreservation also reduced hepatocyte viability, and function and their culture in monolayer downregulates cell receptors involved in cell–cell and cell–ECM interactions, drastically reducing cell functionality over time [87]. The development of 2D culture models such as sandwich culture increased basal and induced drug-metabolizing enzyme activities and simulated in vivo biliary excretion rates [87,88]. However, dedifferentiation of hepatocytes in long-term cultures and the lack of nonparenchymal cells that interact with hepatocytes continued to be inherent disadvantages of these models [89]. The coculture of hepatocytes with other liver cells, such as stellate cells, Kupffer cells, liver sinusoidal endothelial cells, or liver epithelial cells diminishes these limitations to some extent, improving the longevity and functionality of cells and producing the higher expression of CYP and phase

II isoforms compared with monotypic culture [90–93]. Nevertheless, cocultures are usually based on the random mixing of different cell types and thus do not account for their particular anatomical relationship. Looking for more relevant models, to emulate the 3D organization and morphology of hepatocytes within the liver, 3D cultures have been developed. 3D cultures range in complexity from monotypic or heterotypic spheroids [94,95] to 3D scaffold systems [96] and more advanced models using microfluidic in vitro systems [96,97]. Multiple commercial 3D coculture platforms have been developed for drug screening and drug studies such as the "HepatoPac" platform [98], the 3D InSight Human Liver Microtissues of InSphero, the HepaChip in vitro microfluidic system [99], and the HμREL microliver platforms [100].

Although some issues have been addressed for specific applications with these models, others continue to be biologically and technically challenging [88,101].

Organoids in Drug Development

Organoids represent more complex models that try to simulate 3D cell–cell and cell–ECM relationships in more relevant physiological conditions that mimic the liver microenvironment arrangement amenable to the high-throughput screening of compounds feasible enough to guarantee long-term studies.

The optimal liver role depends on the coordinated function of parenchymal and nonparenchymal cells within the hepatic acinus as well as hepatic blood microcirculation. Aspects of the microcirculation can be simulated in vitro via perfusion models to create a dynamic in vivo like environment. Different macroscopic perfused in vitro liver systems initially developed as bioartificial liver devices have been created [97], which provide evidence that perfusion can improve longevity and function in sophisticated hepatic systems, and thus show better in vivo mimicry. Although these models represent the most physiologically practical systems, their size makes them unfeasible for use in drug testing studies because they lack the throughput and analytical flexibility for drug screening. The use of these organoids in drug development involves their miniaturization to a microscopic level. This new class of in vitro tools, often called "on-a-chip" tissue models, can mimic the architecture of small tissue sections and individual characteristics of the dynamic in vivo flow environment, while offering more precise spatial and temporal control of soluble factors. Apart from being amendable to high-throughput screening approaches, these models can be engineered for real-time monitoring of the state of cells and their extracellular environment, which is crucial for determining cellular mechanisms of action in drugs [97].

Several organoid systems have been developed for drug screening and testing. One of the best examples of these organoids is in microfluidic systems. In 2006, Kane et al. developed a microfluidic coculture system of hepatocytes and T3-J2 fibroblasts in an 8×8-well array, demonstrating stable albumin and urea excretion for 32 days. Some years later, HμREL Corporation developed a similar microfluidic in vitro liver platform for drug screening for commercial purposes (HμRELflow) [100]. This platform, formed by multiple fluidically interconnected microscale cell culture compartments, enables simulation of the interaction of test substrates with two or more organs, which provides an enhanced prediction of human response. In fact, in vivo-like absorption, distribution, metabolism, bioaccumulation, and toxicity of naphthalene were demonstrated when lung, adipose, and liver cells were fluidically connected [100]. Furthermore, the size of the system enabled microscopic imaging, oxygen sensing, physiologically appropriate ratios of chamber sizes, hydrodynamic shear stress, and less consumption of media and cells. Even so, some issues such as sample removal, complexity in maintaining recirculation, and cell monolayers on chips and not physiological tissue constructs, significantly limit the model. Some years later, Au et al. developed another microfluidic model, a microfluidic organoid for drug screening (MODS) platform [102]. The novelty of this system compared with the previously developed MODS was the ability to evaluate different conditions simultaneously and the automation of time-consuming processes such as the generation of mixtures and the formation of serial dilution series, which can result in more efficient screening of lead drug candidates. Vernetti et al. developed and characterized a sophisticated system to investigate drug safety and efficacy in liver models of disease. This system includes a human 3D, microfluidic, four-cell, sequentially layered, self-assembly liver model as well as fluorescent protein biosensors for mechanistic readouts and a microphysiology system database to manage, analyze, and model data [103].

Hollow-fiber reactors have also been adapted to drug testing. In 2010, Schelzer et al. developed a microscale prototype of a hollow-fiber reactor. In this model, the bioreactor consisted of four cell chambers, each of which included four compartments (one for cells, two for culture medium, and the last for oxygen supply) connected to provide the cells with a physiologically based environment [104]. The prototype allowed for small numbers of cells and limited reagent use, microscopic evaluation of the cells, and monitoring of oxygen concentrations. Later, a similar system

with coculture of parenchymal and nonparenchymal liver cells was developed for studies of pharmacokinetics and drug toxicity, showing maintained albumin synthesis and CYP activity for 2−3 weeks [105]. Nevertheless, some limitations arise in this kind of systems, such as the lack of physiologic gradients typically seen in liver tissue, the complexity of many tubing lines, and the limited throughput, because only a few different conditions can be assessed simultaneously.

As also mentioned previously, decellularization constitutes a novel approach in liver models [23,25]. This macroscopic model that can be used to investigate liver development and regeneration can also be miniaturized for high-throughput drug studies [106].

Apart from physiological models, organotypic models of liver diseases are being developed for drug testing. Drug metabolism, toxicity, and efficacy in diseased livers differ substantially compared with healthy conditions, so accurate models of disease are required. In this sense, Skardal et al. developed liver-based cell organoids in a rotating-wall vessel bioreactor that inoculated with colon carcinoma cells to generate liver-tumor organoids for in vitro modeling of liver metastasis [65]. Leite et al. developed hepatic organoids with fibrotic features such as HSC activation and collagen secretion and deposition to study drug-induced liver fibrosis [107]. Similarly, Lee et al. generated a reversible- and irreversible-injured alcoholic liver disease model in spheroid-based microfluidic chips in which rat primary hepatocytes and HSCs are cocultured [108].

Although enormous advances have been made to develop more realistic and predictive in vitro liver models for drug testing, the field is still dawning. There are critical issues that should be solved for the field to move forward. Standardizing model and platform characterizations for drug-based studies (viability, secretory capacity, enzymatic and toxicology activities, and drug transporter activity) should be established for each model. Building specificity and sensitivity of the systems, recreating more accurately parenchyma zonation, developing better detection systems and better materials [97], and finding new, unlimited, fully functional cell sources [109] are some challenges in developing in vitro liver models for drug studies.

CONCLUSIONS AND FINAL PERSPECTIVES

This chapter has focused on strategies of liver tissue engineering with potential for developing innovative treatments and efficient models for studying the liver. Knowledge of regenerative biology and medicine has increased exponentially, giving new hope to the development of effective treatments for liver disease. Much work still lies ahead to obtain final therapies or models that accurately represent the liver to its fullest. Standardization of cell isolation protocols and media formulation is needed to achieve a high grade of reproducibility of results among laboratories around the world. Despite the obstacles, some 2D liver culture models, especially cocultures with hepatocytes and nonparenchymal cells (which better represent the in vivo microenvironment of the liver) can be considered useful models for studying the acute phase response, mutagenesis, xenobiotic toxicity, lipid, and drug metabolism in the liver. Liver organoids are also considered a possible tool to assess cell changes that lead to tumorigenesis and cancer progression, and for drug screening and testing. Improvement and standardization of the protocols used are needed to enhance the production rate and ameliorate the features of the obtained organoids.

Regarding the bioreactor systems used in liver tissue engineering, clinical assessment of the efficacy and safety of bioartificial liver systems in treating different end-stage liver diseases is near completion. Then, the field can concentrate on reducing the costs, finding more suitable cell sources, and optimizing bioreactor technologies.

Finally, the use of whole-organ liver scaffolds is promising in the quest of bioengineering a whole liver for transplantation. Livers from different species have been decellularized using various protocols to obtain acellular bioscaffolds. Nonetheless, complete recellularization using all necessary liver cell types, including Kupffer, sinusoidal endothelial, and stellate cells, and the generation of a fully functional liver have not yet been accomplished. Hence, one of the most significant challenges in whole-organ liver bioengineering is an appropriate cell source to repopulate the acellular scaffold and achieve well-defined complete revascularization of the decellularized liver. Induced pluripotent stem cell (iPSC) technology has the potential to provide a source of cells for whole-liver bioengineering for humans. However, bioengineering a fully functional organ with a size comparable to that of humans has yet to be achieved by using iPSC technology.

Altogether, the strategies for liver tissue engineering discussed in this chapter may have an impact on innovative personalized tissue engineering-based treatments as alternative and effective therapies for different liver diseases or pathologies.

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CHAPTER

63

Regenerative Medicine in the Cornea

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INTRODUCTION

Structure and Function of the Cornea

The cornea is the transparent front covering of the eye that transmits and focuses light into the eye for vision. It is composed of three main cellular layers: an outermost stratified epithelium, a middle stroma, and an innermost endothelial layer. The total thickness is approximately 550 μm in the center and 750 μm at the periphery [1]. The epithelium of the cornea forms the outer layer and primary protective barrier. It is composed of stratifying, non-keratinized epithelial cells. Epithelial cells also secrete antiinflammatory and antimicrobial factors as an insoluble layer that maintains the tear film [2]. Because cells are lost on the anterior surface of the cornea, new layers of epithelium originate from a basal layer generated by corneal stem cells in the corneo−scleral limbus [3]. The stroma is composed of a hydrated, largely collagenous extracellular matrix (ECM) containing a network of fibroblast-like cells called keratocytes. The single endothelial layer actively osmoregulates the entire structure and maintains hydration while pumping out excess fluid. The cornea is avascular and relies on its extensive network of sensory neurons and their interaction with the corneal cells to maintain tissue integrity and heal wounds [4].

The external location of the cornea makes it prone to injury and infection. Diseases of the cornea are the fourth largest cause of vision loss globally according to the World Health Organization [5]. Corneal blindness is treated by transplantation with donated human corneas, but there is a severe worldwide shortage of good-quality corneas. Not taking into consideration patients in poorly served remote areas or those contraindicated for conventional surgery, an estimated 12.7 million patients are awaiting corneal transplantation globally [6]. This need is particularly elevated in low- to middle-income countries, e.g., China and India, where 2 million and 7 million patients, respectively, are awaiting surgery.

Treatment Options, State of the Art, and Need for Corneal Regenerative Medicine

The only widespread treatment is corneal transplantation using a full-thickness human donor cornea through a procedure known as penetrating keratoplasty (PK). PK therefore relies on the ability to access donor corneas via eye banks. In 2012, there were 184,576 corneal transplants in 116 countries globally [6]. This means that of 70 patients needing transplantation only 1 is treated, and this is mainly because of a severe global shortage of human donor corneas. However, even with adequate access to donated tissues, although initial graft success rates are high in developed nations, e.g., 90% over 2 years in Sweden [7], only about 64% of grafts survive over 10 years [8]. In developing nations where there is a preponderance of severe pathologies that place patients at high risk for rejection, the graft survival rate over 2 years was 52% [9].

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Corneal prostheses known as keratoprostheses (KPros) have been developed mainly as alternatives to donor corneas for high-risk grafts, because these patients are generally contraindicated or not prioritized for conventional donor transplantation in countries where there is a severe donor shortage. The traditional KPro that is used clinically is composed of an optical core, often made from poly(methyl methacrylate) and a skirt that interfaces with the patient's eye. The two most widely used KPros (the Boston KPro and osteo-odonto-keratoprosthesis) and their variants contain a biological interface. However, the irreversible nature of the surgery, potential complications, and the need for sustained antibiotics and immune suppression cause them to remain an option for end-stage eyes, so they are not ideal replacements for donor human corneas [10]. For more detailed reviews of KPros, see Avadhanam et al. [10]and Salvador-Culla et al. [11].

Regeneration of the corneal epithelium from cell-culture expanded limbal epithelial stem cells pioneered by Pelligrini et al. was a major breakthrough in modern corneal blindness treatment and is in clinical application as the first European Commission approved cell-based advanced therapy medicinal product (ATMP) [12,13]. A direct method for limbal cell replacement known as simple limbal epithelial transplantation (SLET) that bypasses stem cell expansion has also entered clinical application [14,15]. However, limbal stem transplantation is most successful when using an autograft, and in cases where the pathology involves deeper layers, follow-on transplantation with a donated cornea is still needed [16].

The first successful regeneration of the cornea using a cell-free method was reported by Griffith and colleagues and resulted from the use of recombinant human collagen (RHCIII) hydrogels [17]. Ten patients underwent the implantation of proregeneration scaffolds by anterior lamellar keratoplasty [17]. Patients were given partial-thickness implants composed of RHCIII cross-linked using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide–*N*-hydroxysuccinimide, resulting in the regeneration of the epithelium, stroma, and corneal nerves (Fig. 63.1). Because the neocorneas were derived from the patients' own cells growing into cell-free scaffolds, there were no immune problems and patients did not require immune suppression beyond the 6–7 weeks of local steroid eye drops prescribed prophylactically.

Regenerative medicine remains the main option for restoring the structure and function of the cornea to the semblance of a normal cornea. In this chapter, we review current methods used to regenerate the human cornea. Because of the wide variety of methods used, we will review only examples that are in clinical evaluation and are being evaluated preclinically, as well as some novel methods.

REGENERATIVE MEDICINE APPLIED TO KERATOPROSTHESIS DEVELOPMENT

KPros under development and clinical evaluation incorporate elements allowing regeneration. Specifically, devices have been designed to allow the regrowth of corneal epithelium to cover the device to maintain the tear film and prevent infection or extrusion of the implant. This is done by surface modification of the interface opposing the patient's corneal tissues with either surface chemistries or lithographic techniques that pattern the surface on a nanoscale to microscale.

The macromolecules of ECM proteins such as collagen, fibronectin, laminin, their derivative cell adhesive peptides (e.g., isoleucine–lysine–alanine–valine, Tyr–Ile–Gly–Ser–Arg [YIGSR], and Arg–Gly–Asp [RGD]), or other ECM analogs have been evaluated for their ability to modulate epithelial growth over KPros. Previous work has shown that corneal epithelial cell adhesion and growth were significantly enhanced by tethering of laminin or fibronectin adhesion promoting peptide via flexible polyethylene glycol (PEG) chains, more so than by tethering of fibronectin or simple coating of the surface with matrix proteins [18,19]. Modification with fibronectin-based Arg–Gly–Asp–Ser (RGDS) [20–22], laminin-based YIGSR [23,24], and a novel collagen-based peptide, Gly–Pro–Leu [25], was also shown to improve epithelial cell adhesion in vitro. Surface modification peptide combinations, e.g., well-known cell adhesion peptides RGDS and YIGSR together with their synergistic counterparts Pro–His–Ser–Arg–Asn and Pro–Asp–Ser–Gly–Arg, have resulted in surfaces that provide improved corneal epithelial cell adhesion and growth [23].

Growth factors such as epidermal growth factor or insulin-like growth factor-1 have been tethered onto polydimethylsiloxane and polymethacrylic acid-*co*-2-hydroxyethyl methacrylate, respectively [18,26]. Both studies showed that the growth factors enhanced corneal epithelial cell attachment and growth and that the use of a PEG tether has been shown to improve cell coverage of the polymers significantly in vitro. Both sets of results strongly suggested that the spacer molecules provided the correct microenvironment for the epithelial cells by exposing the bioactive motifs to allow the cells to reach confluence, compared with little or no epithelial growth on the surfaces that were coated only with the bioactive factors.

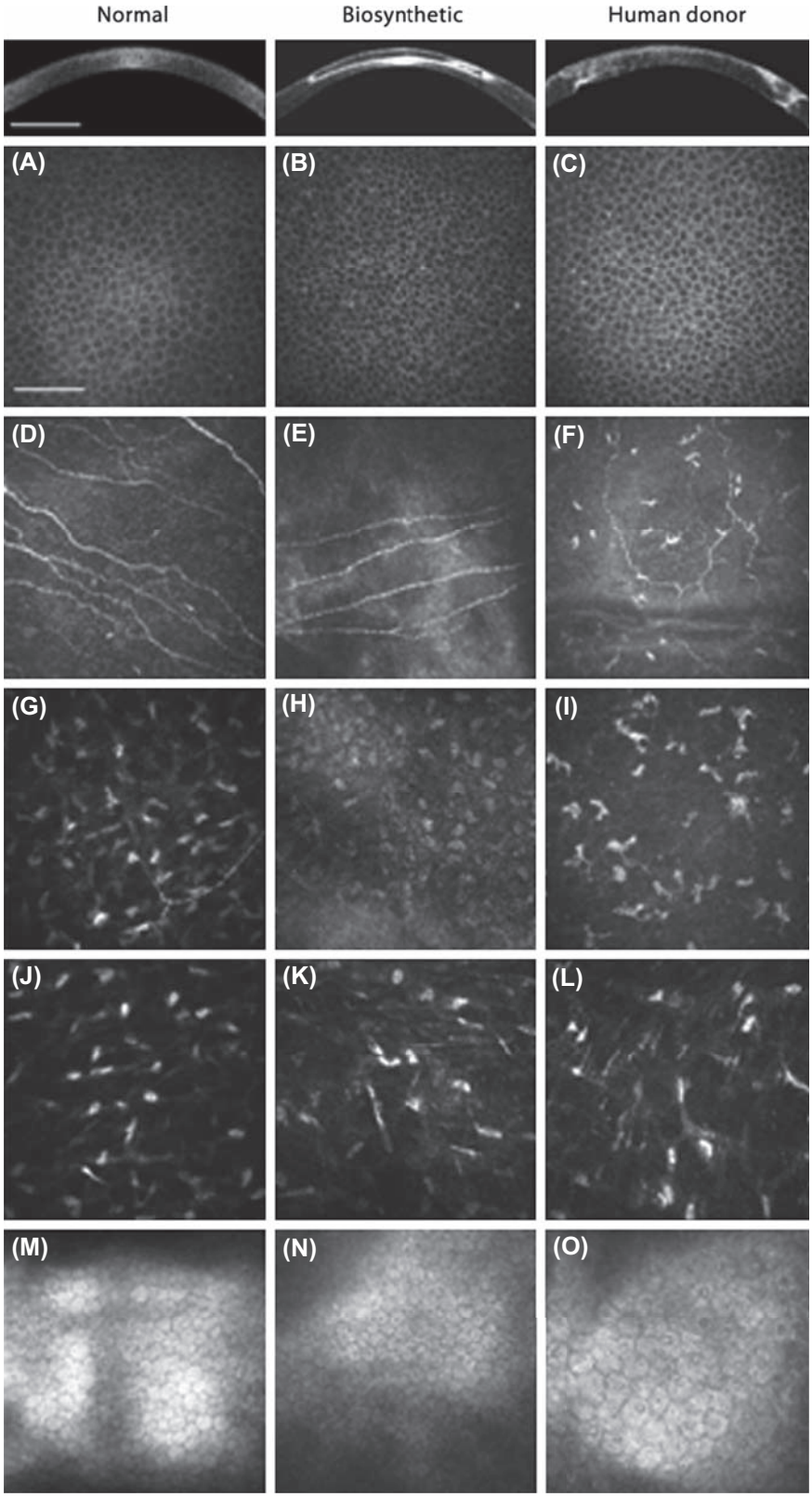

FIGURE 63.1 Corneal features in a healthy, unoperated subject, alongside those of operated patients, at 24 months after implantation of a biosynthetic cornea or a human donor cornea. (Top row) Anterior segment optical coherence tomography (ASOCT) images of a healthy cornea, biosynthetic implant, and human donor transplant by penetrating keratoplasty. Areas of wound-healing activity exhibit high reflectivity (*white areas*). (A–O) In vivo confocal microscopy (IVCM) images. Intact epithelium of the unoperated cornea (A), regenerated corneal epithelial cells on the implant surface (B), and regenerated epithelium of the penetrating graft (C). Regenerated nerves (E) at the subbasal epithelium in an implanted cornea were parallel and morphologically similar to the normal cornea (D), whereas regenerated subbasal nerves were also observed in a cornea transplanted with human donor tissue (F). Anterior stromal cell (keratocyte) nuclei (G–I) and posterior keratocytes (J–L) were present, with varying density, in all corneas. The endothelium (M–O) in all corneas exhibited a characteristic mosaic pattern. *Reproduced from Fagerholm P, Lagali NS, Merrett K, Jackson WB, Munger R, Liu Y, et al. A biosynthetic alternative to human donor tissue for inducing corneal regeneration: 24 month follow-up of a Phase I clinical study. Science Transl Med 2010; 2: 46ra61, courtesy of AAAS.*

Modification of the physical profile of a KPro surface by lithography is another method to enhance the cell–tissue device interface. Surface patterning of a KPro was first reported by Myung et al. using a photolithographically patterned device composed of a PEG–poly(acrylic acid) (PAA) central core and a poly(hydroxyethyl acrylate) microperforated skirt. Type I collagen was then coupled to the hydrogel using photochemical surface modification to promote epithelial migration into the wound site in rabbit models in vitro and in vivo [27]. When the collagen-patterned PEG–PAA hydrogels were implanted into rabbits, the result was good tolerance in 9 of 10 subjects but an optical haze remained at the surgical site [28]. An additional study suggested that the collagen was evenly distributed but cell migration was slower than in surgical controls and took 5 instead of 2.5 days for wound closure [29]. A comparative study of PEG-diacrylate–PAA versus PEG-diacrylamide–PAA hydrogels indicated that the PEG-diacrylamide was more biocompatible than the original formulation [30]. A 2015 study compared porous versus nonporous PEG-diacrylamide–PAA hydrogels with surface modification with type I collagen and fibronectin [31]. The porous hydrogels were synthesized using templates composed of polystyrene microbeads, which were removed after cross-linking by washing in methyl ethyl ketone buffer. Both the porous and nonporous formulations supported the multilayered growth of corneal fibroblasts, which suggested the suitability of porous PEG–PAA hydrogels for KPro skirt material.

REGENERATION OF CORNEAL LAYERS

Corneal Epithelium

Work in regeneration of the corneal epithelium has focused on methods that circumvent the rejection of allogeneic donor cells, because this is a huge problem that necessitates systemic immunosuppression even in patients receiving human leukocyte antigen–matched tissue, which has its own risks and side effects [32]. This includes the development of new therapeutic stem cell sources from transdifferentiation of progenitor cells from other sites such as oral mucosa (or other mucous membranes) to conversion of fibroblasts or mesenchymal stromal/stem cells (MSCs) into corneal lineages by induced pluripotency [33].

Transdifferentiation of oral mucosa has been tested in patients with bilateral corneal epithelial stem cell depletion. In 2004, Nishida et al. reported the first successful human cultivated oral mucosal epithelial transplantation surgeries [34]. The procedure was conducted in four participants with bilateral total limbal stem cell deficiency caused by Stevens–Johnson syndrome (SJS) or ocular cicatricial pemphigoid. The oral mucosal cells were cultured on temperature-responsive cell-culture surfaces with 3T3 feeder cells and transplanted onto one eye of each patient, with successful, stable outcomes. However, all transplanted eyes had some peripheral corneal neovascularization. The procedure was later improved by culture of oral mucosa on human amniotic membrane (HAM) [35]. This technique has been successfully used in patients with SJS, alkali and temperature burns, and aniridia [36–40]. Other stem cells such as umbilical cord lining-derived stem cells have been successfully tested in rabbit models for use in regenerating the corneal surface [41]. MSCs have also been tested as potential sources of corneal limbal epithelial stem cell replacements in rat and rabbit models, but results have been mixed [42–44].

A range of biomaterials have been employed as substrates for corneal epithelial cells for use as implants. Fibrin and HAMs have been the staples. However, materials such as collagen membranes and silk fibroin are being evaluated.

Electrospun poly(D,L-lactide-*co*-glycolide) (PLGA) has also been used as substrate to expand cultured limbal cells and as a human limbal cell explant to restore the stem cell niche [45,46]. PLGA was used at a ratio of 50:50 lactic acid to glycolic acid. The electrospun meshes underwent γ-sterilization and dry storage at −20°C. The meshes degraded at 4–6 weeks in vitro and allowed for the culture of confluent limbal cells. Both cultured and explanted cells generated differentiated and stem cell populations when transplanted into an ex vivo rabbit corneal model.

Corneal Endothelium

The expansion of primary corneal endothelial cells (CECs) in culture before reinjection has been successfully tested in a feline model. CECs were obtained from Descemet membranes excised from feline corneas and expanded for up to two passages after cell detachment. Sixteen animals underwent surgery of the right eye. Eight animals underwent 7-mm central endothelial scraping and injection with 2×10^5 or 1×10^6 cultured CECs, and 100 or 350 μM Rho-associated, coiled-coil containing protein kinase (ROCK) inhibitor (ROCKi). Two underwent

18-mm total scraping followed by intracameral injection of 1 M CECs and ROCKi in 0.2 mL. Six negative controls underwent central or total scraping followed by injection of ROCKi. After surgery, the corneas grafted with CECs performed better than the surgical controls but the endothelium had incomplete functionality and was inferior to the unoperated corneas.

CECs have been differentiated from human embryonic stem cells (hESCs) but in vivo analysis has thus far been limited to animal models. In 2014, Zhang et al. reported human corneal endothelial-like cells derived from the periocular mesenchymal precursor (POMP) phase of hESCs [47]. POMP cells were transferred into coculture with human corneal stromal cells in lens epithelial cell–conditioned media for differentiation into CEC-like cells. The CEC-like cells were sorted using fluorescence activated cell sorting and cultured to generate CEC-like sheets. Transplantation into rabbits undergoing Descemet membrane stripping resulted in gradual restoration of transparency. Two methods of deriving CECs from the neural crest (NC) phase of hESCs have been tested. McCabe et al. derived CEC-like cells from dual Smad inhibitor–generated, feeder-free hESC NC cells with platelet-derived growth factor-β, and Dickkopf-related protein 2 [48]. Song et al. derived CEC-like cells from feeder-supported hESCs differentiated into NC cells cultured in bovine CEC conditional medium and fresh CEC media [49].

Corneal Stroma

Funderburgh and colleagues worked on stroma regeneration and showed that direct injection of stem cells isolated from limbal biopsies can prevent corneal scarring [50]. Limbal stem cell biopsy-derived stromal cells were derived from mesenchymal cells from human cadaveric cornea-sclera rims. In culture, these cells expanded to produce a thick lamellar structure with aligned collagen and proteoglycans resembling the ECM. Grafts in a mouse corneal wound model prevented the formation of scar tissue and were well-tolerated. Corneal stroma sheets have also been constructed in vitro for use as potential replacements for pathologic or thinned stromas. These are discussed subsequently in a discussion on self-assembled corneal constructs.

FULLY CELL-BASED, SELF-ASSEMBLED CORNEAL CONSTRUCTS

Self-assembly of corneal stromal components, largely collagens, has been extensively examined by Germain and colleagues, who used the method of ascorbic acid stimulation of fibroblastic cells to secrete ascorbic acid to prepare a range of tissues. In earlier work, Proulx et al. constructed a corneal stroma by growing stromal fibroblasts in cell culture medium supplemented with ascorbic acid for 28–35 days [51]. The ascorbic acid induces fibroblasts to lay down collagen and other ECM components to form sheets. These 35- to 55-μm-thick sheets are then stacked to form a corneal stroma. After fabrication of a stacked stroma, Proulx et al. added an endothelium and epithelium to construct a three-layered cornea. First, CECs were seeded on top of the reconstructed stroma and allowed to grow into a monolayer in endothelial growth medium. After 2–7 days, a plastic ring was placed on top of the cultured construct that was then turned upside down. Corneal limbal epithelial cells are then seeded on top, after which the entire construct is cultured in epithelial growth medium supplemented with more ascorbic acid. After achieving epithelial confluence, the entire construct is air-lifted and cultured at the air–liquid interface to promote stratification of the corneal epithelium. The resulting cornea was significantly thinner than a native human cornea. However, it was transparent and the authors suggested that full thickness might be achieved by increasing the number of stacked layers of fibroblast–ECM.

A variation on the use of ascorbic acid was reported by Karamichos et al. [52], who employed ascorbic acid with and without transforming growth factor-β to induce elaboration of ECM by human umbilical cord MSCs (cord stem cells) to form a cornea stroma-like structure. The cord stem cells themselves differentiated into stroma-like cells. As reported for the corneal stromal fibroblasts, it took 4 weeks to produce a construct 24 mm in diameter and 30 μm thick. However, in the cord stem cell matrices, 50% fewer cells than fibroblast-produced matrices were present, which showed that the ECM laid down by the cord stem cells was denser.

Self-assembled corneal stromal constructs were tested in animals. The iteration of self-assembled corneal stromal was reported by Syed-Picard et al. [53]. Human cornea stromal stem cells were seeded onto micropatterned PDMS substrates supplemented with stem cell growth medium. After 48 h, the medium was switched to a keratocyte differentiation medium containing ascorbic acid, fibroblast growth factor-2, and transforming growth factor-β3. After 10 days, a sheet was produced that could be detached from the PDMS substrate. After 5 weeks of implantation into stromal pockets in the corneas of immune-competent mice, the sheets were

well-integrated with persistence of human donor cells. They had also acquired transparency during the implantation period (Fig. 63.2).

Thicker sheets can be produced using a similar method, by stacking of self-assembled stromal constructs. Corneal stromal constructs were generated from both feline and human donor corneas and six sheets of stromal cells were stacked to generate each graft, which was then implanted into the corneas of cats in stromal pockets [54]. The animals each received two 4-mm-long and 300-μM-long incisions poles apart in the superior nasal and inferior temporal quadrants, after which a graft was placed in each intrastromal pocket. Four animals received human xenografts and four animals received feline allografts. Contralateral eyes underwent surgery without grafting. Preoperatively, the grafts were slightly hazy but they were restored to complete transparency after day 37. The grafts remained transparent and avascular with reinnervation and showed normal corneal sensitivity (Fig. 63.3). Overall, the surgery was well-tolerated with no symptoms of immune rejection.

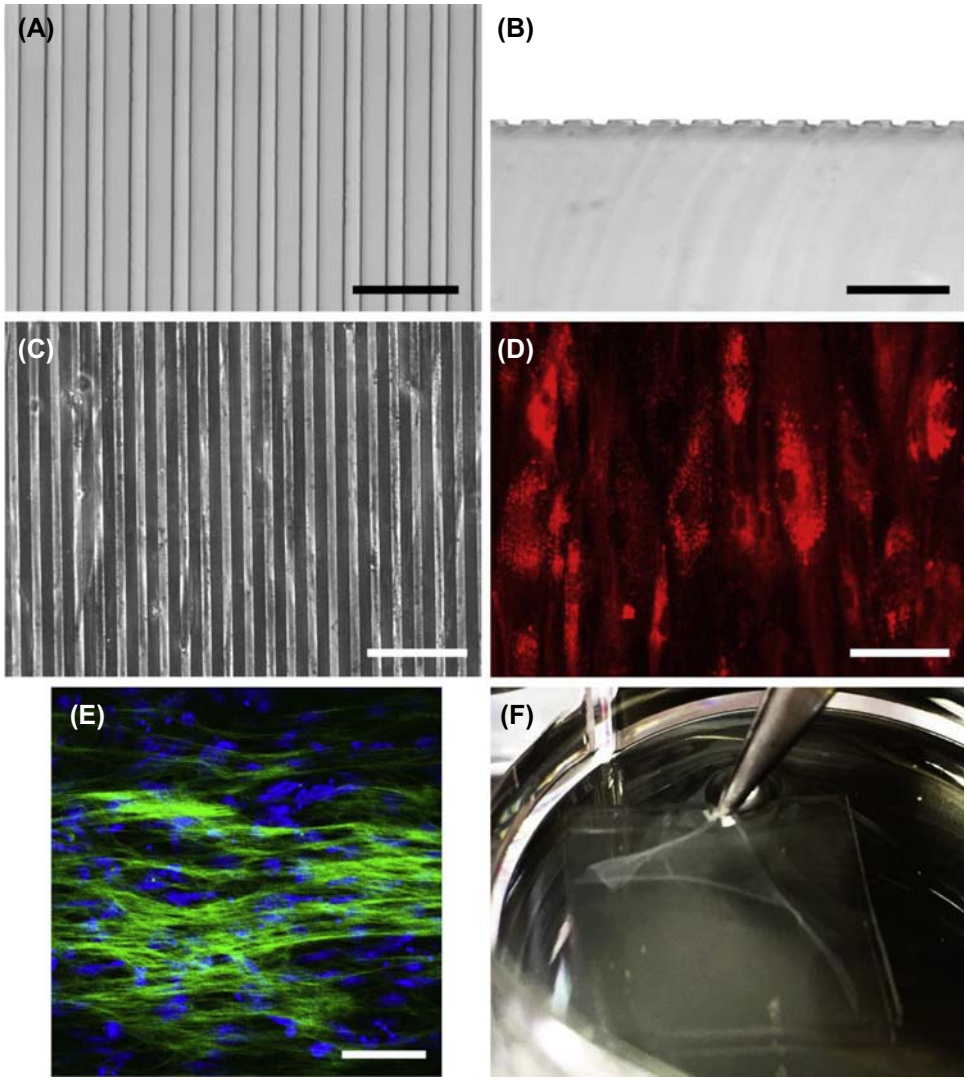

FIGURE 63.2 Formation of scaffold-free tissue sheet with parallel cell and matrix organization. Light micrographs of (A) top view and (B) cross-sectional view of the polydimethylsiloxane substrate show grooves approximately 10 μm wide, 10 μm apart, and 5 μm deep. (C) Phase contrast image shows corneal stromal stem cells (CSSC) cultured on the grooved substrate. (D) For better visualization, CSSC were labeled with DiI (*red*) and cultured on grooved substrate. (E) Two-photon micrograph of 10-day cultures of CSSC on grooved substrates in keratocyte differentiation medium shows deposition of parallel organized collagenous matrix (*green*). Nuclei (*blue*) were stained by SYTOX-green (*blue*). (F) After 10 days of culture, a robust tissue sheet is formed that can be separated from the substrate using forceps. Scale bars: (A) and (B) = 50 μm; (C)–(E) = 100 μm. *Reproduced from Syed-Picard FNN, Du YY, Hertsenberg AJJ, Palchesko RR, Funderburgh MLL, Feinberg AWW, et al. Scaffold-free tissue engineering of functional corneal stromal tissue. J Tissue Eng Regen Med 2016;12(1):59–69, with permission from Wiley and J. Funderburgh.*

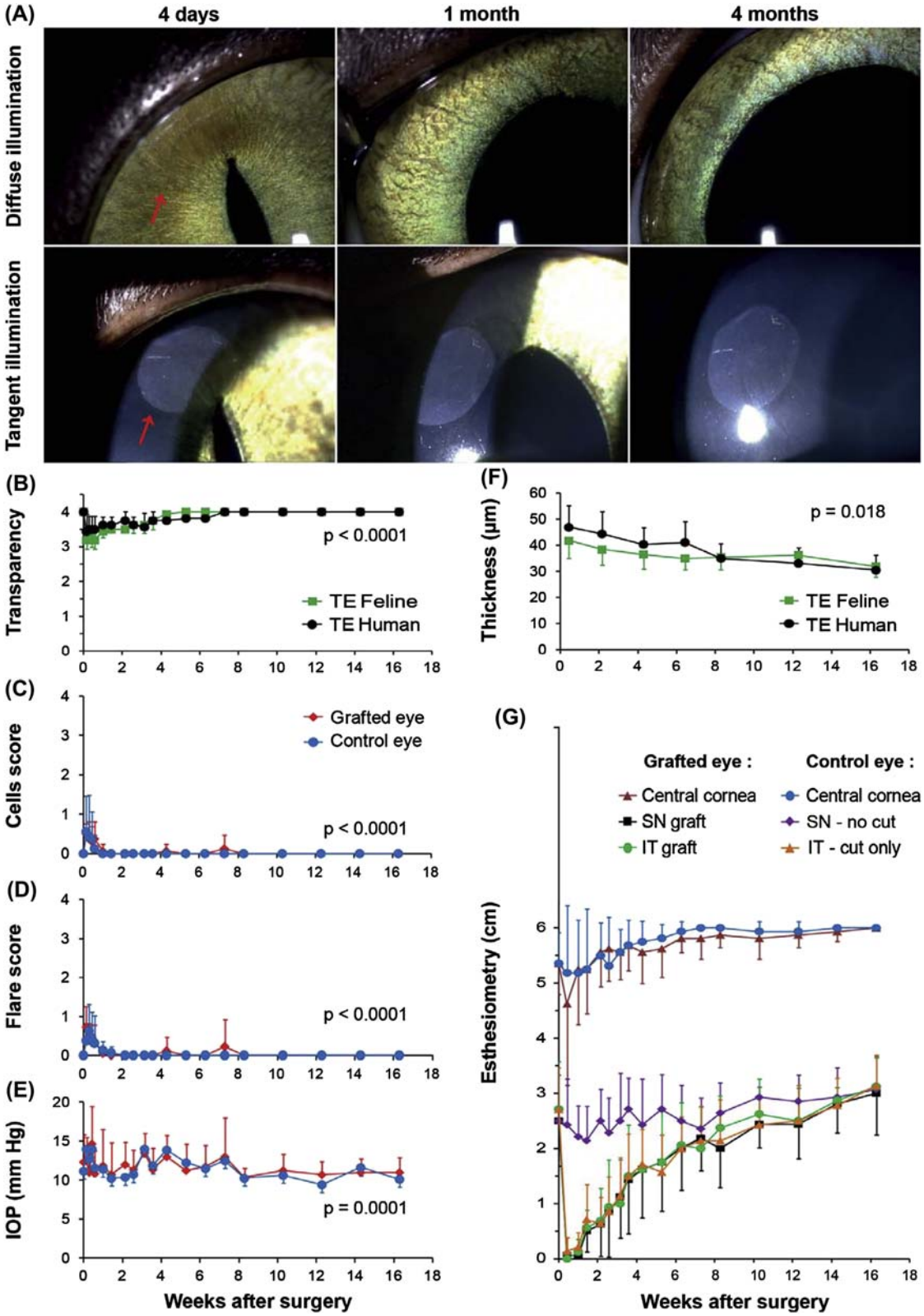

FIGURE 63.3 Clinical outcome of the tissue engineered (TE)—intrastromal grafts. (A) Representative slit-lamp photos at 4 days (column 1), 1 month (column 2), and 4 months (column 3) after transplantation of a TE-stroma in the superior nasal quadrant. Regular diffuse illumination (first row) does not allow visualization of the clear graft, which can only be seen with direct tangential illumination (second row; *arrow*; same graft and ×10 magnification for all six photographs). (B—G) Clinical evolution of grafted and control eyes. (B) Transparency score. (C) Anterior chamber cell score. (D) Anterior chamber flare score. (E) Intraocular pressure (IOP). (F) Graft thickness. (G) Esthesiometry. The parameters described in this figure were measured in all eyes at all indicated time points, with no missing data. The most representative photos are shown. *SN graft*, superior nasal graft; *IT graft*, inferior temporal graft. *Reproduced from Boulze Pankert M, Goyer B, Zaguia F, Bareille M, Perron MC, Liu X, et al. Biocompatibility and functionality of a tissue-engineered living corneal stroma transplanted in the feline eye. Invest Ophthalmol Vis Sci 2014;55(10):6908—20.*

Self-assembled stromal constructs therefore show promise for regenerative medicine applications; however, they have some significant limitations. Allogeneic cells may be problematic in clinical applications, so patient-derived autografts are more likely to be preferred. This poses significant surgical limitations, because patients will need to undergo surgery to isolate the donor tissue and then wait a significant period for the fabrication of a construct. This type of custom construct will then be limited to clinics with on-site cleanroom facilities. The possibility remains of decellularizing the stromal ECM and implanting it, but this has been attempted only in cartilage [55].

CELL-FREE BIOMATERIALS

A range of ECM-based or inspired proregeneration scaffolds have been proposed as alternatives to the largely collagenous corneal stroma. These range from human and xenogeneic decellularized ECM (from pig corneas to fish scales) to matrices made from extracted human and animal collagen, RHC, or short peptide analogs of ECM proteins.

Decellularized Extracellular Matrix as Implants

The aim of decellularization is to remove the cellular content while preserving the native ECM that is believed to promote regeneration when it is used as cell-free or pre−cell seeded implants. A wide range of decellularizing methods have been tested, including agents such as enzymes, chelating agents, chemicals (acid and alkali treatment and alcohols), detergents, hypertonic and hypotonic solutions, and physical methods (freezing, pressure, sonication, and mechanical agitation) [56]. Automated devices for decellularization have also been developed [57].

In 2011, Daoud et al. reported the clinical evaluation results of 150 patients who received anterior lamellar keratoplasty with human decellularized corneas sterilized with γ-radiation [58]. These implants showed high levels of epithelialization within a few days and no postoperative infection or rejection in all but four cases, all of which occurred in patients with preexisting corneal melting. In 2015, Zhang et al. described the implantation of decellularized porcine corneal stromas into the corneas of 47 patients who had corneal fungal ulcers [59]. They reported that 41 of the implants became transparent over time and 34 patients (72%) showed vision improvement. These results are promising, but caution must be exercised for xenogeneic transplantation because severe allergic reactions have been reported [60,61]. The use of poor-quality cadaveric human corneas as decellularized implants instead of living allografts is still caused by cornea shortage problems and requires confirmation of safety, because immunogenicity and risk of disease transmission remain considerations.

The main structural protein in decellularized ECM is collagen. The scales of fish comprise 41−81% of connective tissue protein and collagen and have been examined as collagen implants. Emergency patients with perforated corneal ulcerations or lacerations referred for immediate treatment at European hospitals often receive a temporary human cadaveric donor cornea. This so-called “0-cornea” is used to close the eye only temporarily because it does not meet all requirements of human donor corneas used for PK. However, an “0-cornea” is not always immediately available; hence, the ologen Biocornea was developed (Fig. 63.4) This is a temporary cornea made from decellularized and decalcified fish scales, designed to seal perforated corneas temporarily for up to 72 h while waiting for a donor human cornea to become available. When tested in rat corneas [62], these fish

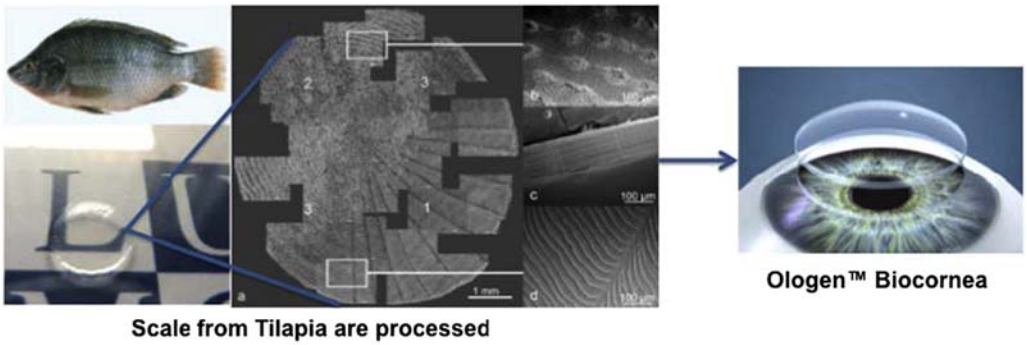

FIGURE 63.4 Ologen Biocornea derived from fish scale has a morphology similar to human cornea extracellular matrix and is transparent. *Courtesy of Aeon Astron Europe B.V.*

scale–derived collagen matrices were not reepithelialized during the 3-week study period and integration was incomplete in two of six animals, leading to the loss of implants. Some melting was also observed. As implants within stromal pockets, the grafts resulted in haziness. The ologen Biocornea was also reported to be able to seal full-thickness cornea perforations in minipig models in which there was no leakage of aqueous humor, the anterior chamber of the eye retained its normal depth, and there was only mild swelling of the wounded cornea [63]. Histopathological examination of the patched cornea showed that the temporary repair did not trigger immune cell invasion and prevented epithelial ingrowths into the wound.

An important consideration for the use of xenoderived, decellularized cornea stromas is the heterogeneity of the decellularization process and the risk for allergic reactions to xenogeneic biomaterials (compared with allogeneic biomaterials) and pathogen transmission if tight processing controls are not adhered to [64]. China Regenerative Medicine International is moving ahead with the industrial-scale use of decellularized pig corneas as alternatives to human donor corneas [65].

Collagen-Based Implants

The 4-year follow-up of the first successful regeneration of multiple cell types in the human cornea showed that implants made from carbodiimide-cross-linked RHCIII were able to engraft stably without immune suppression [66]. Interestingly, even at 4 years after the operation, the regeneration process in several patients appeared to be ongoing. Histopathology of a graft from a patient who elected to be regrafted (owing to a problem with contact lenses needed to correct astigmatism resulting from suturing) showed that the regenerated neocornea had a structure similar to that of a normal, healthy cornea.

For corneas with inflammation or more severe pathologies such as chemical burn, however, implants made from RHCIII alone were not able to prevent neovascularization, as shown in rabbit models of alkali burn [67]. The incorporation of a network of 2-methacryloyloxyethyl phosphorylcholine (MPC), a synthetic lipid with known inflammation suppressing properties [68] properties, blocked neovascularization [69]. When the RHCII–MPC implants were grafted into three patients as tectonic patches for relief of symptoms from chronic corneal ulceration from burns and a previously rejected graft, i.e., individuals with severe pathologies causing pain, severe discomfort, and photophobia who were diagnosed to be at high risk for rejecting conventional donor corneas, RHCIII–MPC implants restored surface integrity and relieved the patients of the symptoms [69]. Two of the three patients also showed improvement in vision. A more recent clinical study of RHCIII-MPC implants as anterior lamellar grafts in six patients over an average of 24 months showed stable restoration of surface integrity, relief of symptoms in patients with ulceration, and improvement in touch sensitivity indicating regeneration of corneal nerves [70].

In Islam et al. patterning RHCIII–MPC hydrogel constructs with microcontact printing, another form of soft lithography, showed that different widths of fibronectin stripes were able to modulate cell adhesion and proliferation characteristics [71].

Peptide Analogs of Extracellular Matrix

Full-length ECM macromolecules are often hard to extract in large quantities and to purify. Animal source material has the risk of pathogen transmission and requires screening before use in humans [72]. In addition, these macromolecules are relatively difficult to functionalize compared with synthetic polymers. Hence, the development and use of short analogs of ECM components would allow for more effective processing and applications. Peptide analogs have been tested for the fabrication of scaffolds.

Peptide amphiphiles (PAs) containing the Arg–Gly–Asp (RGD) cell adhesion motif from fibronectin were developed by Miotto et al. to promote corneal regeneration (PAs) [73]. When assembled into film coatings, such PAs enhanced the adhesion, proliferation, and alignment of human corneal stromal fibroblasts while inducing the formation of three-dimensional (3D) lamellar-like stromal tissue [74]. Uznalli et al. tested PAs based on laminin peptides [75]. When used as injectable scaffolds in rabbit models of corneal wound healing, they reported an increase in keratocyte migration into the surgically induced wounds that led to enhanced stromal regeneration. In addition, PAs using matrix metalloproteinase sequences were developed that self-detach after reaching confluency [76,77]. Finally, a PA based on lumican that forms nanotape structures was reported to support human corneal fibroblasts and increase collagen production [78].

Islam et al. reported the use of a short peptide analog of collagen in fabricating corneal implants [79]. A sequence from O'Leary et al. [80] conjugated to multiarm PEG through thiol-maleimide chemistry through a "C-G" peptide linker resulted in a hybrid hydrogel consisting of the collagen-like peptide (CLP) and PEG. CLP–PEG hydrogels

were successfully tested as a proregeneration scaffold in corneas of minipigs as anterior lamellar grafts. Regeneration of corneal epithelium, stroma, and nerves were recorded over the 12-month observation period (Fig. 63.5). The timing and similarity of the regeneration were comparable to those of control implants made from RHCIII−MPC previously tested in human patients [69,70].

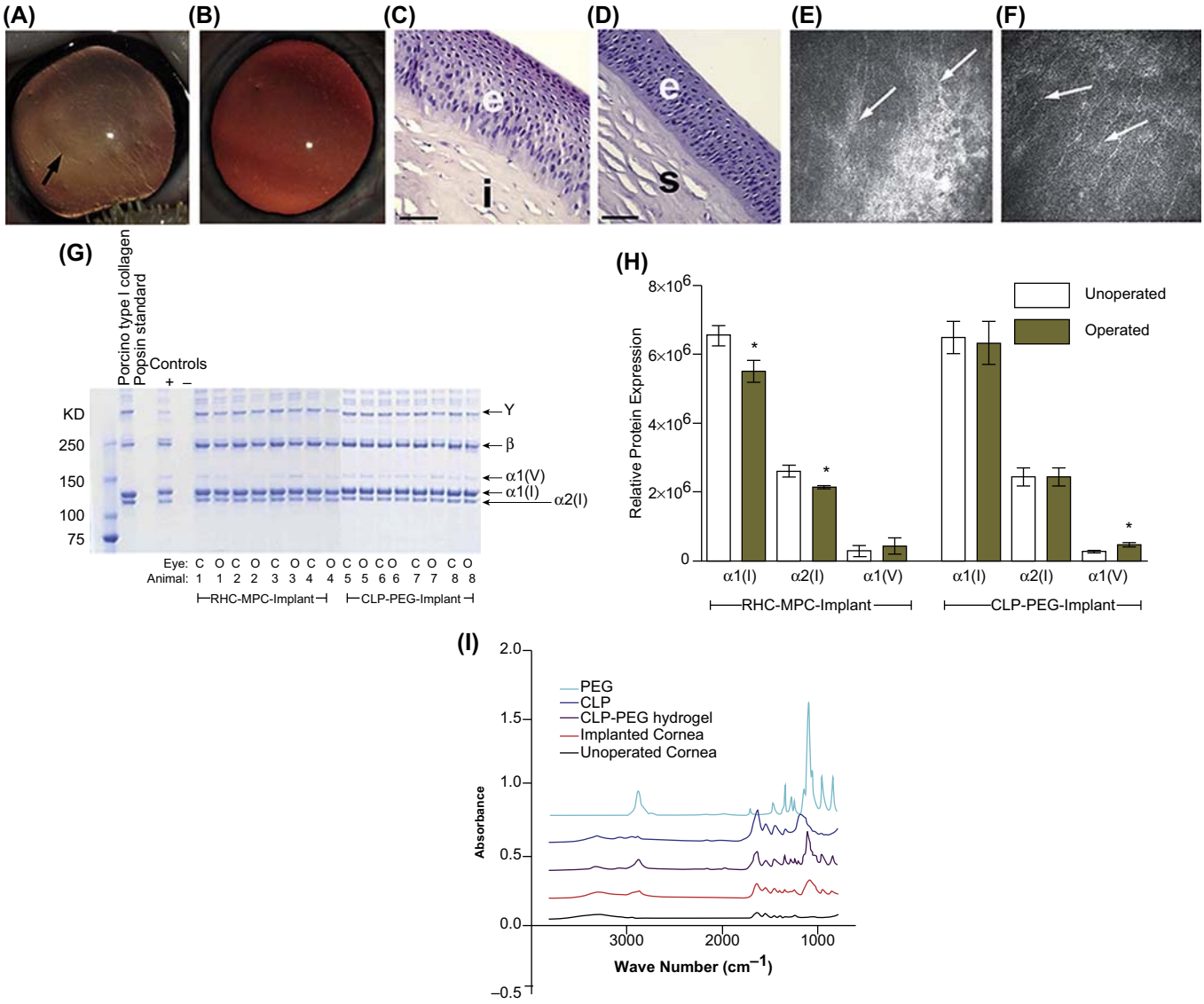

FIGURE 63.5 Postsurgical corneal regeneration at 12 months after collagen-like peptide (CLP)−polyethylene glycol (PEG) implantation. (A) Optically clear CLP−PEG implant (*arrow*) stably integrated within the pig cornea, compared with its healthy, unoperated contralateral cornea (B). Blood vessels are seen in the implanted cornea but stop at the margin of the implant. (C) Hematoxylin-eosin staining of a representative regenerated CLP−PEG neocornea compared with a healthy control cornea (D) showing similar morphology. Scale bars = 50 mm. (E) In vivo confocal microscopy shows the regenerated nerve (*arrows*) in CLP−PEG cornea that follow a parallel pattern similar to that of the unoperated cornea (F). (G) Sodium dodecyl sulfate−polyacrylamide gel electrophoresis separated proteins from the central cornea area of both implanted and control corneas show the presence of α1 and α2 chains for type I collagen (α1[I] and α2[I], respectively) and α1 chain for type V (α1[V]), in the regenerated CLP−PEG implanted cornea, similar to recombinant human collagen (RHC)-2-methacryloyloxyethyl phosphorylcholine (MPC) and the unoperated healthy control corneas. (H) Analysis of normalized, relative protein content shows that the levels of α1(I) and α2(I) in CLP−PEG implanted corneas were similar to those of the control healthy corneas (no statistical significance) whereas the level of α1(V) was higher than that of the control (*$P < 0.05$). In RHC-MPC−implanted corneas, levels of both α1(I) and α2(I) were significantly lower than in the unoperated controls (*) whereas α1(V) were similar. No differences were observed between RHCIII-MPC and CLP−PEG implants in general. (I) Fourier transform infrared analysis of regenerated pig neocorneas 12 months after grafting with CLP−PEG implants shows the presence of both CLP and PEG. *e*, epithelium; *i*, implant; *s*, stroma. *Reproduced from Islam MM, Ravichandran R, Olsen D, Ljunggren MK, Fagerholm P, Lee CJ, et al. Self-assembled collagen-like-peptide implants as alternatives to human donor corneal transplantation. RSC Adv 2016;6(61):55745−9.*

CELL–BIOMATERIAL COMPOSITES

Cell–biomaterial composites have also been proposed as artificial corneas. Collagen has been used to develop composites of epithelium and stroma from isolated stem cells, e.g., as Real Architecture for 3D Tissue equivalents. A first-in-human study is under way.

Silk, particularly the structural protein fibroin derived from the domesticated silkworm *Bombyx mori*, has been explored as potential corneal stromal scaffolds by several groups in conjunction with therapeutic stem cells. Several examples are discussed here. Groove-patterned, RGD-functionalized silk substrates have been fabricated and seeded with human corneal stromal stem cells (hCSSCs) and human corneal fibroblasts (hCFs) as potential human corneal stromas [81]. The hCSSCs effectively differentiated into stromal cells that elaborated a cornea-specific ECM to mimic human corneal stromal tissue. These constructs were 90–100 μm thick and contained ECM components such as collagen, keratin sulfate, lumican, and keratocan. On the other hand, the hCFs differentiated into myofibroblasts that deposited less-organized collagen in a pattern resembling that of corneal scar tissue. In 2016, Wang et al. reported the successful implantation of helicoidal multilamellar RDG functionalized silk fibroin films in rabbits [82]. At 180 days after the operation, the implants were transparent without signs of neovascularization or immunogenic response. Wang et al. reported the construction of a 3D corneal model using silk with endothelium, stroma, and innervation supported on a free-floating PDMS scaffold in culture media [83], which allowed an air–liquid interface. This and previous models using collagen showed that an air–liquid interface enable optimal corneal cell and nerve ingrowth in multilayered corneal constructs [84–86].

Silk fibroin has also been blended with chitosan to form scaffolds [87]. These were seeded with cornea stromal fibroblasts to form constructs that were implanted into the corneas of 15 rabbits for 12 weeks to allow regeneration. The regenerated rabbit corneas were described as being comparable to healthy, unoperated corneas, with expression of K3/12 expression in the corneal epithelial cells and vimentin in the stromal cells.

Hazra et al. reported the creation of a silk fibroin film generated from non–mulberry feeding silkworms, *Antheraea mylitta* (Am) [88]. The Am film supported the proliferation and differentiation of limbal stem cells. After implantation in rabbits, the films were clear and well-tolerated without neovascularization at 2-month follow-up.

COMPOSITE IMPLANTS INCORPORATING SPECIFIC BIOACTIVE FUNCTIONS

Infections are an important cause of vision loss and corneal blindness [2]. Hence, implants that contain nanoparticles designed to combat microorganisms or viruses have been under development. Collagen-based implants incorporating antibiotics (vancomycin) or nanoparticles releasing the antiviral drug acyclovir have been tested and found to be effective at blocking the activity of *Staphylococcus aureus* in rabbit infection models and herpes simplex virus serotype 1 (HSV-1), respectively, in vitro [89,90]. A variant on implants with antiviral properties is a collagen-based implant that incorporates corneal cells engineered by gene transfer to secrete the innate antiviral cathelicidin peptide, LL-37 [91]. The secreted LL-37 was able to inhibit viral binding and reduce the incidence of plaque formation and plaque size in vitro but was insufficient to protect cells completely from HSV-1 infection.

Silver nanoparticles (AgNPs) have been reported to have broad antiinfective and antiinflammatory properties. Collagen implants incorporating AgNPs have also been developed, along with a strategy to prestabilize and incorporate AgNPs with different colors into collagen matrices to fabricate corneal implants of different colors and antimicrobial properties [92].

CHALLENGES

From a regulatory perspective, regenerative implants and grafts in the cornea are primarily classified as medical devices or ATMPs, respectively. The most straightforward type of implant to manufacture is a fully synthetic scaffold, especially one that is thermostable and can be sterilized by γ-irradiation or electron beam. These scaffolds do not have to contend with rules concerning xenografts. Scaffold derived from animals, either through decellularization or by isolating structural proteins, requires more testing to comply with requirements to prevent pathogen transmission. The most complex regulatory submissions are cell and scaffold composites, which are regulated under the ATMP guidelines. Pellegrini et al. successfully initiated the first application for an ATMP

product in Europe, but this remains an area in which there are many safety considerations and few examples to guide both the applicant and the regulatory agencies [93]. It will likely remain incumbent on the applicants to demonstrate that their product is effective, reproducible, and sterile. Extensive risk analysis of all phases of the production and implantation of an ATMP will be required to generate sufficient confidence that the product will be safe for an extended market.

CONCLUSIONS AND FUTURE PERSPECTIVE

There is a wide variety of solutions for regenerating the cornea. Most recently, 3D bioprinting was used to print corneal stromas complete with cells [94]. Progress has been made in cell-based therapies, ECM-based scaffolds, nanomaterials, and combinations of advancements in cell and biomaterials. The challenge to replace human donor corneas and KPros as the current standard of care remains complex. Advances in cell-based therapies have significantly improved the probable efficacy of corneal autografts, and biomaterials have improved the structural properties of ECM-based scaffolds; however, no single therapy has emerged as a stable, affordable solution for the many causes of corneal damage. It is possible that in the future, biomaterial scaffolds may be combined with surgical techniques such as SLET or cultured cell solutions to customize commercially available scaffolds for individual patients. Nanomedicine also opens the door to the possibility of incorporating therapies for eye infections directly into surgical solutions, which may speed postoperative recovery and prevent latent infection recurrence in patients with corneal blindness caused by infections such as HSV.

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CHAPTER

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Alimentary Tract

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INTRODUCTION

The alimentary tract is a hollow organ that starts at the mouth and terminates at the anus. It conducts a number of highly complex and diverse functions that are regulated by distinct cellular and functional differences along the tract length, which allow it to provide the body with nutrients, water, and electrolytes. To achieve this, it is essential that the lumenal contents be propelled along at a rate that will allow efficient digestion and absorption to take place while also enabling waste products to be stored and excreted in a controlled manner. In addition to this, an important symbiotic relationship exists between bacterial species that colonize the alimentary tract of the host [1]. Therefore, the surface of the alimentary tract must also provide a barrier against unwanted entry of organisms and toxins. If the barrier function is breached, specialized cells and tissues within the gut wall provide an important component of the immune system to protect the host.

Dysfunction of the alimentary tract may result from a variety of congenital and acquired conditions that can affect any of its physiological functions. This chapter will discuss knowledge regarding tissue engineering different regions of the alimentary tract, highlighting successful strategies as well as failures and some obstacles that have yet to be overcome in this rapidly evolving field.

ESOPHAGUS

The esophagus is a muscular tube measuring approximately 25 cm in length in adult humans. It functions primarily as a conduit to connect the pharynx with the stomach, providing coordinated peristaltic contractions in response to swallowing to propel food into the stomach. The esophageal mucosa is lined by stratified, squamous, nonkeratinized epithelium. The submucosa contains muscle, nerve, blood vessels, lymphatics, and mucosal glands. The muscularis has two layers consisting of an outer longitudinal layer and an inner circular layer. Both layers consist of striated muscle in the upper portion and smooth muscle in the lower third, continuous with the muscle layers of the stomach. The myenteric plexus exists between the muscle layers. The esophagus has no serosa and its vascular supply is less extensive compared with the intraabdominal portions of the gut. Sphincters at the upper and lower ends of the esophagus ensure food is transferred appropriately between it and the pharynx or stomach. The upper esophageal sphincter, found in the upper 3–4 cm of the esophagus, and the lower esophageal sphincter, located 2–5 cm above the gastroesophageal junction, remain tonically and strongly constricted to prevent air from entering the esophagus during respiration between swallowing and reflux of stomach contents into the esophagus between peristaltic waves, respectively. Regenerative medicine techniques are being explored for a number of conditions affecting the esophagus.

Gastroesophageal reflux disease is one of the most common disorders affecting the gastrointestinal tract, resulting from lower esophageal sphincter incompetence. Medical therapy is generally safe and effective in most cases, but for patients for whom this option fails, antireflux surgery or endoscopic procedures that involve injecting bulking materials may be used in an attempt to narrow the lumen of the lower esophagus. Attempts have been made to restore physiological function of the esophagus using regenerative medicine. The feasibility of using a suspension of muscle precursor cells to restore gastroesophageal function in a model of gastroesophageal reflux disease has been explored

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[2]. Muscle precursor cells isolated from expanded satellite cells derived from skeletal muscle fibres were injected into the gastroesophageal junction after cryoinjury. Histology showed an increase in myofibers at the site of injection that had fused into newly formed or preexisting myofibers. The need remains to demonstrate that cells injected in this manner can contribute to functional improvement of damaged esophageal sphincter, but the feasibility of using this approach offers a promising therapy for this common condition.

Esophageal reconstruction is a requirement for congenital esophageal atresia, burns, malignancy, or severe benign disease. Surgical techniques include stretching, circular myotomy, and interposition of stomach or colon, but these approaches are frequently associated with complications including stricture, leakage, elongation, and gastroesophageal reflux. An artificial esophageal construct has been sought for many years. To be effective, the construct must be implantable without rejection and biocompatible to support appropriate tissue growth, and retain biomechanical characteristics of native esophageal tissue, i.e., be soft and elastomeric while maintaining a tubular structure when implanted in vivo.

Attempts to tissue engineer replacement esophageal tissue have included both patch and circumferential implantation of constructs composed of synthetic as well as natural scaffold materials. A full-length of tissue engineered esophagus has not been produced, but a number of incremental advances toward this goal have been achieved.

Early attempts exploring the use of a nondegradable prosthetic tubes in canine models were of limited success [3]. Surgical reconstruction techniques of the esophagus after resection for structures and malignancies have involved the transfer of small segments or patches of skin and other tissues on a vascular pedicle, with moderate results [4–6]. Tissue engineered sheets of autologous oral mucosal epithelial cells were successfully transplanted by endoscopy in a canine model [7]. The transplanted sheets adhered to the underlying esophageal muscle layers created by endoscopic submucosal dissection and enhanced wound healing without postoperative stenosis. Because the interaction between the epithelium and mesenchymal cells is thought to reduce fibrosis and scarring that cause stenosis, the investigators suggested that this approach may offer a novel therapy to reduce scarring and prevent painful constriction that can be associated with endoscopic submucosal dissection for the removal of large esophageal cancers.

A variety of scaffold materials to support cell and tissue esophageal constructs have been investigated [8–12]. Acellular scaffolds composed of extracellular matrix components have been explored primarily because they are assumed to be advantageous over synthetic scaffold materials owing to their ability to promote cell attachment, growth, and cell–cell signaling among different tissue components. Decellularized esophageal tissue can be produced via repeated detergent-enzymatic treatment that results in a scaffold with biocompatibility suitable for the growth of esophageal epithelial cells [13,14]. Based on these preclinical findings, it has been envisaged that human donor esophageal tissue might one day be used in a manner similar to that described for the tissue engineering of human airway tissue [15].

Scaffolds derived from small intestinal submucosa (SIS) have been widely investigated for tissue engineering replacement esophageal constructs. SIS consists of extracellular matrix material harvested from porcine small intestine and has been used extensively in tissue engineering experiments since it was originally described by Matsumoto and colleagues in 1966 for use in large vein replacement in dogs [16–20]. It has been successfully applied to regenerative medicine applications in humans, including repair of hernias, diaphragms, and tympanic membranes, and for large wound coverage [21–24].

The success with using SIS as a scaffold to promote tissue regeneration appears to relate to the retention of collagen (types I, II, and V), growth factors (transforming growth factor, fibroblast growth factor 2, and vascular endothelial growth factor), glycosaminoglycans (hyaluronic acid, chondroitin sulfate, and heparin sulfate), proteoglycans, and glycoproteins (fibronectin) during the fabrication process [25,26]. It is thought that the resulting scaffold has a composition closely resembling native extracellular matrix, which makes it ideally suited for the attachment and growth of new tissue.

The extent of circumferential replacement of esophageal tissue appears to have an impact on the outcome of attempts to tissue engineer esophagus, with patches producing better results compared with tubular segments. Lopes and colleagues successfully used SIS patches to repair defects to the anterior wall of cervical or abdominal esophagus in rats without signs of stenosis over 150 days [27]. Likewise, Badylak and colleagues used SIS patches (or urinary bladder submucosa) to repair esophageal defects created in dogs without clinical signs of esophageal dysfunction [28]. However, the latter study reported signs of stenosis in dogs receiving complete circumferential segmental grafts of SIS [10]. Doede and colleagues reported similar findings, with severe stenosis occurring when relatively short tubular lengths (4 cm) of SIS were used in alloplastic esophageal replacement in piglets [28]. Thus, although scaffolds consisting of only extracellular matrix have shown the capacity to promote cell growth in vitro and tissue regeneration of patch defects in vivo, replacement of circumferential defects without stricture

formation remains difficult to achieve. The outcome of repairing full circumferential defects using SIS may be improved by optimizing the poor mechanical properties of the scaffold material. To address this, hybrid scaffolds composed of SIS combined with synthetic polyesters (poly[3-hydroxybutyrate-*co*-3-hydroxyhexanoate] and poly [lactide-*co*-glycolide]) were assessed for their feasibility as a tissue engineering scaffold for esophageal constructs [29]. Improved biocompatibility was observed with the hybrid scaffold compared with scaffolds produced from the synthetic material alone.

Epithelial-mesenchymal cell signaling is likely to have an important role in facilitating reconstruction of the esophageal construct after implantation. A similar effect has been shown in bladder reconstruction, in which the presence of urothelium led to infiltration of fibroblasts into acellular matrices and apparent transdifferentiation into a smooth muscle phenotype [30]. Signaling from the mesenchymal cell population appears to be equally important in promoting growth of overlying epithelium [31]. Complete reepithelialization with little inflammatory response and evidence of skeletal muscle regeneration was observed when bone marrow mesenchymal stem cells were seeded onto the SIS scaffold implanted in a canine model [32]. Moreover, the presence of epithelial-mesenchymal signaling may prevent stricture formation in an esophageal construct, a problem frequently encountered with many of the scaffolds tested [10,33]. Similar signaling properties have been demonstrated in bladder reconstruction in which acellular collagen scaffolds seeded with urothelium and smooth muscle cells prevented tissue contraction [34]. Likewise, the interaction of muscle with the ablumenal surface of esophageal scaffolds at the time of implantation of partially circumferential grafts appears to have accounted for the reduced stricture formation observed in a canine model of esophageal reconstruction described by Badylak and colleagues [11]. It can be concluded from these observations that careful consideration of the order in which cells are added to the tissue engineered construct will improve the likelihood of achieving a successful outcome.

In addition to SIS, gastric acellular matrix was used as scaffold by Urita and colleagues to regenerate esophagus in a rat model [35]. Grafts of gastric acellular matrix were used to patch defects in the abdominal esophagus and animals were killed at points between 1 week and 18 months. Although regeneration of the muscle layer or lamina muscularis did not occur, there was no evidence of stenosis or dilatation at the graft site. The matrix obtained in this study was from whole stomachs, but the investigators suggested that gastric acellular matrix may provide an autologous source of naturally derived extracellular matrix scaffold in a clinical setting, because the portion of stomach destroyed to obtain the matrix is minimal. It remains to be seen whether this approach is feasible in a larger animal model, but the use of autologous acellular matrix scaffolds avoids concerns related to the use of xenogenic scaffold materials such as porcine-derived SIS. In addition to the risk of transmitting viral pathogens and prions, cultural and religious beliefs may need to be considered when using acellular matrix scaffolds derived from certain species. In addition to SIS, other biological materials have been investigated for esophageal tissue engineering. Extracellular matrix scaffold has been generated from ovine forestomach tissue [36]. Moving away from mammalian sources of scaffold material, Franck and colleagues reported that a bilayer silk fibroin matrix composed of porous silk fibroin foam annealed to a homogeneous silk fibroin film exhibited improved cell attachment and spontaneous differentiation of esophageal epithelial cells toward a suprabasal cell lineage compared with SIS scaffolds [37].

Although the esophagus can be considered one of the less complex regions in the alimentary tract, several significant hurdles still need to be overcome before tissue engineering and clinical replacement of full-length esophageal segments become a clinical reality in humans. Unlike patch grafts, replacement of longer lengths of tissue will be unable to rely on adjacent esophagus to cover the surface area of larger scaffolds via guided tissue regeneration. Improved methods from isolating and expanding the different esophageal cell populations will therefore be a prerequisite for successful tissue engineering of larger constructs. Kofler and colleagues identified subsets of ovine esophageal epithelial cells that may help achieve this [38]. PCK-26−positive esophagus epithelial cells demonstrated high proliferative capacity and uniform coverage on collagen scaffolds, which the investigators suggested could have an important role for successfully tissue engineering esophagus. Further refinement of the scaffold material may also improve epithelialization. For example, the inclusion of copper into acellular porcine SIS scaffolds was reported to enhance epithelialization of the scaffold in a canine model of esophageal repair [39].

Failure to regenerate a functioning muscle layer may not be problematic for short or noncircumferential grafts, but for longer lengths of esophagus the presence of an innervated functional muscle layer will be essential. A retrospective study investigating the temporal appearance and spatial distribution of nervous tissue in a canine model of esophageal reconstruction using porcine urinary bladder submucosa showed the presence of nerve tissue within sites of the remodeling scaffold [40]. Although the study was unable to demonstrate whether the nervous tissue was functional or to distinguish among the various subsets of neurons, it opens the possibility of using similar models to identify mechanisms that promote innervation that will facilitate the tissue engineering of functional tissue. Peristalsis of food also depends on the correct orientation of muscle fibers in the wall of the alimentary tract.

To address this, promising results have been obtained with orienting smooth muscle tissue on unidirectional scaffolds for tissue engineered esophagus in rats [41]. Oriented stands of smooth muscle mimicking the configurations found in the native organ were engineered when cells were seeded onto unidirectional scaffolds. These were assembled with esophageal epithelium to create a hybrid approach.

STOMACH

Gastric disease affects approximately 10% of the world's population and includes gastritis, peptic ulcers, and gastric cancer. Insufficient stomach mass, which may arise from gastrectomy or congenital microgastria, is associated with increased patient morbidity.

The stomach functions as a digestive organ and reservoir and is anatomically divided into four regions (cardiac, fundus, corpus, and pylorus). The gastric glands are tubular structures whose cellular composition and function are specialized according to each region of the stomach. Such specialization, combined with the harsh environment created by the lumenal contents, makes tissue engineering of the stomach as whole challenging. The size and shape of the stomach vary, depending on its contents. The stomach wall contains outer longitudinal and inner circular layers of smooth muscle, with an innermost layer of oblique muscle fibers. These layers facilitate important functions including storage of ingested food in the stomach until it can be accommodated in the lower portion of the alimentary tract, mixing of the food to form chyme, and regulation of food transit into the small intestine at an optimal rate for digestion and absorption. Stomach emptying is controlled by the gastric food volume and the release of the hormone gastrin, as well as feedback signals from the duodenum.

Tissue engineered neostomach constructs to patch partial gastrectomy have been explored in a canine model using a two-part sheet composed of an outer layer of collagen sponge and a temporary inner silicone sheet to protect the collagen from degradation by the acidic stomach juices and provide mechanical support [42]. After removal of the silicone sheet at 4 weeks, evidence of stomach regeneration was observed and complete coverage of the scaffold had occurred by 16 weeks, confirmed by the presence of mucosa and a thin muscular layer. Acid production capacity was present in the regenerated stomach wall but the contractile response to acetylcholine was poor [43]. Technical difficulties associated with suturing and endoscopic removal of the silicone sheet in this model were addressed by creating a tissue engineered sheet without silicone that had sufficient strength to allow suturing and resist anastomotic dehiscence [44]. The silicone sheet was replaced by a biodegradable copolymer of poly(D,L-lactide) and ε-caprolactone (PDLCL) on the mucosal side of the collagen scaffold, both of which were completely absorbed at 16 weeks' implantation. Although regeneration of the stomach mucosa was observed, the replacement of the silicone sheet with PDLCL did not provide sufficient mechanical strength to prevent significant shrinkage of the scaffold.

The feasibility of creating new stomach tissue using stomach-derived organoid units harvested from neonatal and adult rats has been investigated [45]. The organoid units were seeded onto polymer scaffold tubes to form constructs that were implanted into the omentum of adult syngeneic rats. At 4 weeks, the construct was anastomosed to the small intestine. Histology of the tissue engineered stomach tissue was similar to native stomach, with gastric pits, squamous epithelium, and positive staining for α-actin smooth muscle in the muscularis and gastrin indicating the presence of a well-developed gastric epithelium. The same approach has been used to tissue engineer stomach neoconstructs in an autologous large animal model [46].

A limitation of approaches using autologous or allogenic organoid units is the tissue source. To address this, three-dimensional gastric organoid tissue from human pluripotent stem cells (PSCs) have been generated by temporal manipulation of the fibroblast growth, WNT, bone morphogenetic protein, retinoic acid and epidermal growth factor (EGF) signaling pathways [47]. The primitive gastric organoids exhibited molecular and morphogenetic developmental stages similar to those observed in the developing antrum of murine stomach. The proliferative zones contained $LGR5^{+}$ cells, mucus-secreting antral and pylorus cells, and gastric endocrine cells; however, the acid secreting (corpus) region was not developed in this model. A critical factor for the development of all the stomach regions from stem cells appears to be the Barx-1 gene [48]. Noguchi and colleagues demonstrated Barx-1—inducing culture conditions generated spheroids of fully functional stomach-like tissue in vitro from mouse embryonic stem cells. The spheroids were able to develop into functional corpus and antrum tissue that secreted pepsinogen and acid.

The relevance of mesenchymal stem cells to optimize and condition the cellular milieu within the tissue engineered construct has also been shown with stomach tissue engineering. Tissue regeneration after the creation of a full-thickness stomach defect in rats was enhanced when SIS scaffolds were used in conjunction with mesenchymal

stem cells [49]. Whereas contractility in response to a muscarinic receptor agonist, a nitric oxide precursor, or electrical field stimulation was observed in all groups, smooth muscle layers were both longer and better structured compared with SIS grafts not seeded with mesenchymal stem cells.

SMALL INTESTINE

The small intestine in adults measures approximately 6 m in length from the duodenojejunal flexure to the ileocecal valve. Its primary function is absorption of nutrients from the lumen, a process facilitated by specialized mucosal surface features (folds of Kerckring, villi, and microvilli) that increase the absorptive surface area about 600-fold to approximately 250 m^2. The mucosa is lined with epithelium overlying lamina propria containing vascular and reticular stroma, large aggregates of lymphoid tissue called Peyer patches, and a strip of smooth muscle called the muscularis mucosa. Intestinal stem cells reside at the base of epithelial invaginations into the mucosa called crypts and develop into all four lineages of epithelial cells that line the intestine [50]. Epithelial cells migrate along the crypt–villus axis, differentiating and maturing toward the lumen of the bowel where they become senescent over the course of a few days and are shed into the lumen of the bowel.

A lack of intestinal epithelial stem cell markers has hampered identification and isolation of pure populations of cells for regenerative medicine purposes. Studies have shown that Musashi-1 may be a marker of intestinal stem cells [51,52], and a Sox9-(enhanced green fluorescent protein [EGFP]) mouse model has been used to enrich multipotent intestinal epithelial stem cells [53]. Using a culture system that mimics the native intestinal epithelial stem cell niche, these cells are capable of generating "organoids" that contain all four epithelial cell types of the small intestinal epithelium. Furthermore, Sox9-EGFP multipotent intestinal epithelial stem cells express CD24, which may facilitate their enrichment by fluorescence activated cell sorting using widely available antibodies.

The submucosa consists of fibrous connective tissue that supplies blood and lymphatic vessels to the mucosa. The muscularis propria consists of an inner layer of circular muscle and an outer longitudinal muscle layer. The muscularis propria is covered by the adventitia, a layer of loose connective tissue, and the serosa, a mesothelial lining of peritoneum.

The function of the small intestine cannot be replaced by transposing another part of the gut. Intestinal ischemia and bowel resection for tumors and inflammatory bowel disease can result in short bowel syndrome when more than 75% of the small intestine is lost. Short bowel syndrome is often associated with intestinal failure and the requirement of lifelong nutritional support (total parenteral nutrition), which is frequently accompanied by severe complications such as liver failure, line sepsis, and poor long-term survival rates. The length of residual intestine is critical for these patients; thus, techniques for increasing absorptive surface area have been sought for many years. Surgical options for increasing the absorptive surface or slowing the transit time to enhance absorption have been reported, but these approaches require longer residual intestinal segments and most have only limited long-term clinical success [54–57]. Small bowel transplantation is a viable option for some patients, but this procedure has limitations including the availability of donor tissue, the need for long-term immunosuppression, graft versus host disease, and potential posttransplant lymphoproliferative disorder [58].

The amount of small bowel required for successful nutritional rehabilitation depends on factors including the patient's age, the amount of small bowel present, the presence or absence of the ileocecal valve, and the amount of large bowel present. Therefore, small bowel elongation of just a few centimeters could allow many patients to become independent of total parenteral nutrition. Distraction enterogenesis has been devised as a novel method to increase intestinal length by applying linearly directed force, resulting in increased surface area and epithelial cell proliferation [59,60]. Devices used for distraction enterogenesis include extralumenal, radially self-expanding shape memory polymer cylinders [61]; biodegradable springs composed of polycaprolactone created to lengthen intestinal segments mechanically while avoiding the need for subsequent retrieval [62]; and double-balloon catheter devices [63]. Biological mechanisms that account for distraction enterogenesis are unknown [64], but the effect can be enhanced by adding exogenous glucagon-like peptide 2 [65] or by codelivering microspheres that provide sustained release of basic fibroblast growth factor (bFGF), which results in improved vascularity [66]. The use of growth factor–embedded scaffold materials is an effective method for improving the short half-lives of growth factors. An example used to achieve growth factor–embedded scaffolds for distraction enterogenesis include subcritical CO_2 to embed heparin-binding EGF-like growth factor into polyglycolic acid/poly-L-lactic acid scaffolds [67]. Local delivery of the trophic growth factor improved structure of the tissue engineered intestine.

Several different approaches, using either guided tissue regeneration or tissue-engineering neotissue constructs, were taken to regenerate small intestine that used combinations of various synthetic and natural scaffold materials, different cell types, and surgical procedures.

Early attempts to patch bowel defects using the serosal surface of another piece of intestine resulted in its being covered with regenerated mucosa [68,69]. This paved the way for other researchers to investigate a variety of scaffold materials, such as polytetrafluoroethylene tubing, for the ingrowth of neointestine via guided tissue regeneration [70]. The use of nonresorbable materials for studying intestinal morphogenesis and regeneration continues to be interesting [71], but the use of resorbable scaffold biomaterials for intestinal tissue engineering has become the predominant approach.

Chen and Badylak used SIS to patch partial defects in the small bowel wall of a canine model [72]. Histological evaluation showed the presence of mucosa, varying amounts of smooth muscle, sheets of collagen, and an outer serosal layer. However, in the same study, attempts to use a tubular configuration of SIS were unsuccessful. The tubes either leaked or became obstructed; this occurred primarily because the SIS was unable to maintain lumenal patency when exposed to the moist lumenal contents. Similar limitations with the mechanical integrity of the scaffold material were reported by Pahari and colleagues, who used guided tissue regeneration to create a segment of new intestine in rats using acellular dermal matrix (AlloDerm) rolled into tubes [73]. Building on the work, elongation of the intestine was achieved using an acellular biologic scaffold to create autologous bioartificial intestinal segments (BIS) [74]. The BIS were demonstrated to have functional absorptive characteristics [75]. Other approaches using biologically derived scaffolds included the use of allogenic aortic graft segments interposed in an excluded small bowel segment wrapped in omentum, which resulted in intestinal-like wall transformation of the aortic graft [76].

In an attempt to maintain an open lumen in the tissue engineered intestine, Hori and colleagues reported that scaffolds composed of sheets of acellular collagen sponge wrapped on a temporary silicone stent and covered with omentum guided tissue regeneration of almost all layers of the gastrointestinal tract in a canine model, but only a thin muscularis mucosa was present and the muscularis propria was absent [77]. The same group explored the addition of mesenchymal stem cells seeded onto a collagen scaffold, which it was hypothesized might differentiate "site-specifically" into muscle cells and regenerate the muscle layer [78]. Intestinal regeneration occurred but muscle regeneration in an organized manner was not observed. Wang and colleagues used a rat model to evaluate the feasibility of regenerating tubular intestine using sheets of rat-derived SIS wrapped around a silicone stent [79]. The tubular graft was interposed in the middle of a Thiry-Vella loop (a defunctionalized segment of ileum that is brought out as a double ileostomy) in Lewis rats. The silicone stent was left in place for 3 weeks to maintain lumenal patency during tissue regeneration. At 4 weeks, an epithelial layer had begun to form and completely covered the lumenal surface by 12 weeks. The neomucosa had a typical morphology containing goblet cells, Paneth cells, enterocytes, and enteroendocrine cells. Although the regenerated bowel contained bundles of smooth muscle-like cells, especially near the sites of anastomosis, the quantity and organization of the muscle layer differed from those found in native small intestine; they were predominantly circular muscle with no longitudinal muscle. The use of a Thiry-Vella loop in the model created by Wang may have facilitated mucosal development in the neointestine by protecting it from alimentary transit and creating an isolated environment that avoided the food stream and digestive enzymes. Lee and colleagues observed only minimal intestinal regeneration in a rat model used to evaluate SIS scaffolds. From this, they concluded that SIS scaffolds alone were insufficient to regenerate small intestine and suggested that the use of appropriate progenitor cells is probably necessary to facilitate the regeneration of small intestine [80].

Many of the studies reporting intestinal tissue engineering strategies are based on methodologies used in the pioneering work conducted by Vacanti and colleagues in the 1980s and 1990s that combined intestinal tissue with scaffolds [81]. Important to these studies were previous investigations by Tait and colleagues that showed intestinal tissue could be separated by enzymatic digestion to produce organoid units [82]. These clusters of cells contained all of the elements of the intestinal mucosa including stem cells and mesenchyme, which could be used to regenerate intestinal neomucosa expressing digestive enzyme activities and glucose transport capacity similar to those of age-matched native intestinal mucosa. When organoid units were subcutaneously grafted, they displayed different epithelial populations consistent with epithelial transit amplifying and stem cell populations [83].

Subsequent studies demonstrated that transplanting organoid units onto biodegradable polymer scaffolds followed by implantation into the omentum of syngeneic adult animals resulted in the formation of neointestinal cysts attached to a vascular pedicle with mucosa facing a lumen that contained mucoid material [84]. The mucosa of the neointestine created with this technique showed morphological similarities to native intestine, including the formation of a primitive crypt–villus axis lined with columnar epithelial cells and goblet cells and a polarized epithelium with the brush border enzyme sucrase expressed at the apical surface and laminin at the basolateral surface, and

transepithelial resistance similar to that of native intestine [85]. For all of these promising findings, it is essential that the tissue engineered construct facilitates nutrient absorption. Anastomosis of the tissue engineered cyst-like structures to native jejunum in adult rats provided continuity with the native intestinal tract, which resulted in a more developed neomucosa containing significant increases in villus number, villus height, and surface length of the cyst compared with nonanastomosed cysts [86]. The investigators postulated that anastomosis may have facilitated neomucosal growth in the cysts by draining lumenal contents or via stimulatory factors present in the lumenal contents of the native intestine in continuity with the neomucosa. The anastomosed neointestine was also shown to express of Na^+-dependent glucose transporter SGLT1 [87] and a mucosal immune system with intraepithelial and lamina propria immune cells similar to that of native jejunum [88]. The native small intestine has a great adaptive and compensatory capacity in response to massive small bowel resection, which is considered to be controlled by humoral factors. The mucosa of the neointestine was also shown to possess this adaptive capacity after massive small bowel resection, resulting in a significant regenerative stimulus for the morphogenesis and differentiation of the tissue engineered intestine [89]. An improvement in intestinal function, capable of facilitating patient recovery after massive small bowel resection, was putatively demonstrated when cysts containing neointestine were anastomosed to native small bowel during an 85% enterectomy in rats [90]. The study showed that animals with tissue engineered intestine returned to their preoperative weight more rapidly compared with animals undergoing small bowel resection alone. These findings significant because they are the first to suggest that tissue engineered intestine may provide a therapeutic intervention for managing patients with short bowel syndrome. Although it is tempting to speculate that the observed effects resulted from neointestine restoring absorptive function after small bowel resection, the mechanism underlying the beneficial effects remain uncertain [91]. It has been postulated that the amount of intestine replaced by the anastomosed neointestine (approximately 4 cm) was far shorter than the amount resected, probably approximately 10% of the original length and is unlikely to have added sufficient mucosal surface area to account for the increase in postoperative weight observed. Furthermore, the improved nutrition may have resulted from the tissue engineered intestine slowing intestinal transit, leading to increased absorption and weight gain, a principle that could be achieved with simpler remedial surgical procedures [91]. To gain a better understanding of the mechanisms underlying the formation of neointestine, the model has been transitioned from a rat to a mouse model, which has enabled the use of transgenic tools for lineage tracing. This demonstrated that epithelium, muscularis, nerves, and blood vessels are derived from multicellular organoid units derived from donor small intestines of transgenic mice [92]. Studies also investigated the effects of donor age and region where intestine crypts are harvested [93]. In mice, higher efficiency of enterosphere formation was observed with crypts harvested from tissue collected from the proximal small intestine, and also in young mice.

A significant drawback with this approach is the need for large amounts of donor tissue to harvest a sufficient number of organoid units to seed scaffolds that will generate a relatively short length of neointestine, which is likely to offer only limited therapeutic value [91]. A solution might exist with the use of yet unexplored alternative sources of intestinal epithelial stem cells, such as bone marrow–derived cells and PSCs circulating in the peripheral blood [94,95]. New methods for generating organoid units that address the limitations of harvesting donor intestinal tissue have also been explored. McCracken and colleagues reported a protocol for differentiating human PSCs into three-dimensional (3D) human intestinal tissue, developing into intestinal tissue containing all major types of intestinal epithelial cells and mesenchymal components [96].

To address challenges associated with the limited availability of autologous donor intestine in patients with short bowel syndrome, protocols exist for generating enteroids from minimal quantities of starting material [97]. Also, refinement of the harvested donor stem cells or manipulation of growth factors in the local environment may provide a method for enhancing the quality of the neointestine. The morphology of tissue engineered intestine was improved by seeding scaffolds with intestinal stem cell–enriched crypts [98]. Greater circumferential mucosal engraftment and an average villous height closer to native intestine were achieved with the purified crypts collected using a filtration-based system compared with scaffolds seeded with a villous fraction containing differentiated epithelial cells. Likewise, manipulation of the expression of growth factors that control the growth and differentiation of the intestine during development might provide a valuable approach for improving the formation of tissue engineered small intestine. Tissue engineered organoid units overexpressing fibroblast growth factor 10 resulted in larger tissue engineered constructs, with longer villi and a greater proportion of proliferating epithelial cells [99].

Organoid units derived from human postnatal, small bowel resection specimens, were seeded onto biodegradable scaffolds and implanted into nonobese diabetic/severe combined immunodeficiency γ chain–deficient mice [100]. After 4 weeks, the tissue engineered small intestine contained the four major types of differentiated intestinal epithelial cells, muscularis, and intestinal subepithelial myofibroblasts.

Bone marrow–derived epithelial cell adhesion molecule–positive, and CD133-positive cells were used to recellularize human cadaveric small bowel specimens that had been chemically decellularized [101]. After recellularization, the tissue engineered small intestine contained mucin-positive goblet cells, cytokeratin 18–positive epithelial cells in villi, and smooth muscle cells in muscularis mucosa.

Cryopreservation of the organoid units could be used to delay production of tissue engineered small intestine, a procedure that could be particularly helpful in patients who are critically ill and require delayed autologous implantation of a tissue engineered construct. Cryopreservation using vitrification led to higher viability of organoid units compared with standard snap-freezing; the thawed organoid units were capable of producing tissue engineered small intestine [102].

Another important aspect of intestinal tissue engineering is the ability for the neointestine to repair, regenerate, and remodel. The latter is particularly important when considering the use of engineered intestinal tissue for children, in whom the length of the intestine increases significantly during development. The trophic effects of glucagon-like peptide-2 (GLP-2) have been evaluated on neointestinal growth [103]. GLP-2 is an endogenous regulatory peptide with potent trophic effects on intestinal mucosal growth and an ability to modulate the expression of Na^+-glucose cotransporter 1 (SGLT1). Adult rats with neointestinal implants that received subcutaneous injections of a GLP-2 analog twice daily for 10 days had enhanced mucosal growth and increased expression of SGLT1 compared with control rats. These findings indicate that the neointestine is capable of responding to external regulator signals that could be used to further expand the surface of the neointestine.

There is a lack of preclinical models for observing intestinal tissue regeneration and improved intestinal function on a scale that can be feasibly translated into humans. Intestinal tissue engineering has been investigated using a large-animal model designed to emulate conditions required for human therapy [46]. Tissue scaffolds were seeded with organoid units isolated from the jejunum of 6-week-old piglets and implanted into the omentum of that animal. However, the study provided only limited information on issues related to the scaling-up of a technique for use in humans because the neointestine was not anastomosed to the native intestine and the scaffolds used were similar in size to those used in previous small-animal models.

A functional mucosal barrier is an essential element of intestinal tissue engineering for which scalability also needs to be considered. Although transepithelial resistance of the mucosa created in neointestinal cysts is similar to that of native intestine [85], the creation of larger intestinal constructs will require rapid coverage of the scaffold surface to ensure the barrier function is established. This process might be accelerated by including materials in the scaffold that promote epithelial cell spreading. Yoshida and colleagues investigated the effect of transplanting organoid units onto denuded colonic mucosa of syngeneic recipient rats [104]. The addition of bFGF facilitated neomucosal growth and improved restoration of intestinal epithelial cell coverage over the denuded mucosa compared with the control group. Other approaches might include including inorganic materials into hybrid scaffolds, such as bioactive glass, shown to increase epithelial cell migration via bFGF in an indirect manner [105]. As well as stimulating regeneration of the mucosa, delivery of growth factors may provide a strategy for regenerating the muscularis propria. Local delivery of bFGF from scaffolds, via either incorporation into the collagen coating of scaffolds or encapsulation into microspheres, was also shown to increase the engraftment and density of seeded smooth muscle cells and blood vessel formation after 28 days' implantation in the omentum of rats [106].

Rapid vascular in growth into the tissue engineered intestine will be essential to maintain the viability and engraftment of cells seeded on the scaffold. Gardner-Thorpe and colleagues observed that tissue engineered intestine exhibited lower levels of bFGF and vascular endothelial growth factor (VEGF) and a fixed capillary density compared with native juvenile bowel [107]. This led that group to evaluate a polymeric microsphere system to deliver encapsulated VEGF and stimulate angiogenesis in the maturing neointestine [108]. Capillary density in the muscular and connective tissue layers was significantly increased in the presence of microspheres containing VEGF, as were the size and weight of the constructs. Interestingly, the rate of epithelial cell proliferation also increased in constructs implanted with VEGF-releasing microspheres, possibly related to the improved vascularization of the construct providing greater nutritional support to the rapidly proliferating epithelium. The need for neovascularization is not restricted to tissue engineering tissues of the alimentary tract. A number of different approaches are being used to tackle this problem [109]. It remains to be seen whether any of these approaches will provide a sufficient stimulus to promote arteriogenesis required for sufficient vascularization of larger tissue constructs. Furthermore, a functional lymphatic system in the neointestine is essential to establish normal nutrient absorption, fluid homeostasis, and immunological functions. Lymphangiogenesis is reported to occur in the neointestine created by the organoid unit-cyst model in rats [110]. Although angiogenesis has been demonstrated in intestinal tissue engineering using small-animal models, it is not certain whether the provision of thin-walled endothelium lined structures will be sufficient to support the functionality of a larger tissue construct. Therefore,

techniques to promote the formation of medium-sized blood vessels via arteriogenesis are likely to be required to facilitate complete integration of large-scale intestinal constructs with a functional capacity. The small bowel has an extensive vascular system fed by arcades of arteries in the mesentery derived from the superior mesenteric artery. Translation of the existing tissue engineering models to a scale suitable for implantation into humans will require the formation of a similar vascular system consisting of medium-sized blood vessels to maintain viability of a larger tissue construct as well as enable absorption of fluid and dissolved nutrient material from the intestine into the portal blood, which will require a vascular system similar to that found in native intestine. One approach to enabling immediate perfusion of the tissue engineered construct might involve using the existing vascular system in decellularized tissue. Preservation of the vascular structure in decellularized porcine small bowel has been used to engineer tissue via innate vascularization [111]. The decellularized scaffold was repopulated by endothelial cells and exhibited patent vessels after arterial and venous microanastomosis.

Improved methods for seeding and maturing larger tissue engineered intestinal constructs will be needed to ensure that the limited cells available are delivered to the tissue construct efficiently and uniformly. These obstacles may be overcome with the development of bioreactor systems that will assist with the long-term culture and bioengineering of tissues by providing an in vitro environment that is similar to normal physiological conditions. Kim and colleagues designed a perfusion bioreactor specifically for intestinal tissue engineering [112]. The use of bioreactors that provide magnetic force may also be used to deliver cells labeled with superparamagnetic iron oxide nanoparticles into hollow tubular scaffolds with more uniform distribution [113]. Techniques used to tissue engineer vascular grafts might also provide solutions that can be translated to intestinal tissue engineering. For example, centrifugal casting onto decellularized laser-porated natural scaffolds has been reported to enable the rapid fabrication of tubular tissue in a bioreactor-free manner [114].

The type of scaffold material chosen for tissue engineering is an important consideration. An optimal scaffold material must be capable of withstanding the intestinal microenvironment, which poses significant challenges in terms of biocompatibility, mechanical properties, and longevity. It must allow transplanted cells to engraft and proliferate rapidly while enabling tissue perfusion of nutrients and remodeling to ensure complete integration with the host. The composition, geometry, and topography of scaffolds used for intestinal tissue engineering may influence the properties of cells grown on their surface.

Compared with natural extracellular matrix–derived scaffolds, biodegradable synthetic polymer scaffolds provide more control over scaffold properties, such as scaffold architecture, degradation rates, and mechanical properties. Boomer and colleagues evaluated a selection of synthetic tubular scaffolds composed of poly(glycolic acid), poly(ε-caprolactone), poly(L-lactic acid), and polyurethane with either nanofiber or macrofiber structures [115]. Implantation of the scaffolds into the peritoneal cavity of rats revealed different rates of tissue infiltration and scaffold degradation. The inclusion of extracellular matrix components such as hyaluronic acid (a nonsulfated glycosaminoglycan found ubiquitously in connective, epithelial, and neural tissue) was found to enhance the physicochemical properties of gelatin–collagen scaffolds, including attachment, growth, and viability of Caco-2 cells [116]. Histological organization of cells resembling intestinal circular and longitudinal smooth muscle has also been achieved using scaffolds that consist of two layers of orthogonally oriented fibers [117].

The function of the geometry of the crypt–villus microenvironment in regulating intestinal cell proliferation and differentiation was explored by Wang and colleagues [118]. Caco-2 cells migrating over microwell structures showed increased metabolic activity and lower levels of differentiation compared with cells cultured on flat surfaces, which suggested that the structure of crypts may have a role in retaining a proliferative phenotype. Likewise, scaffold architecture is a parameter that can be used to enhance cell infiltration and mass transfer of nutrients to ensure the viability of tissue is maintained. The porosity of the scaffold has a critical role in cell survival and ultimately the viability of the tissue construct. This was illustrated in a study looking at the inclusion of 250-μm pores in multilayered electrospun scaffolds [119]. When implanted in vivo, scaffolds with greater macroporosity were associated with increased blood vessel development and improved survival of intestinal smooth muscle cells, which suggested that macropore connectivity can be optimized to enhance angiogenesis and improve cell viability. Compression molding combined with particulate leaching has been used to create multilayered scaffolds with differential porosities and pore sizes [120]. These structural features were found to influence the behavior and interaction (bridging versus penetration) of different cell types found within the small intestine (epithelial and smooth muscle cells).

The impact of the spatial geometry and mechanics of the microenvironment are likely to have an important role in the physiological functionality of tissue engineered constructs. DiMarco and colleagues used a combination of experimental and finite element analysis to investigate critical variables that control intestinal organoid contraction [121]. Adjustment of ambient oxygen concentration, tailoring the density of the collagen type I matrix, addition of R-spondin1, and culture geometry were found to influence contractile behavior of the organoids. Contractile

behavior occurred only within a narrow range of collagen densities, which the investigators suggested acted as a permissive switch to enable contraction. The inclusion of biomimetic topographical features might provide further control of cell behavior. To replicate the irregular multiscale features of native intestine, Koppes and colleagues created replicas of decellularized porcine small intestine by chemically depositing Parylene C to create molds for polydimethylsiloxane (PDMS) substrates [122]. The PDMS substrates exhibited multiscale folds, crypt and villus structures, and submicron features of the basement membrane. Finer control over the impact of microenvironmental cues is likely to improve scaffold performance, which affects tissue formation, development, differentiation, and functional behavior. Culture of primary intestinal organoids in recombinantly engineered extracellular matrix allowed improved physical manipulation of the scaffolds biomechanical cues while retaining the efficiency of organoid formation to match that obtained with natural collagen I matrices [123]. This provided a means to optimize the properties of the scaffolds to achieve improved matrix performance and identification of microenvironmental cues crucial for bioengineering tissue constructs. Porous protein scaffold systems composed of silk fibroin have also been used to replicate the tissue architecture and microenvironments of the intestine [124]. This type of insight into how properties of the scaffold materials influence the functional behavior of cells is crucial if physiological features such as the stem cell niche, crypt−villus axis, and peristalsis are to be achieved in the tissue engineered construct.

Combined with the revolution in additive manufacturing, an icreased understanding of the biochemical and mechanical cues that control tissue regeneration will lead to a step change in state-of-the-art technology available to create scaffolds tailored to replicate the native scaffold of tissues in terms of topographical, mechanical, and biochemical features. Lee and colleagues fabricated scaffolds with a high surface area−to-volume ratio using 3D printing technology [125]. The growth of smooth muscle cells in vitro was found to be influenced by the geometry of the scaffold. Scaffolds with small villi (0.5 mm) had increased cell density compared with scaffolds containing large villi (1 mm) after 14 days of culture. A 3D printable Matrigel−agarose system was described for the printing of intestinal epithelial cells [126]. The agarose component provided mechanical stability for the 3D printed structure and the Matrigel provides essential protein components for cell growth and spreading. The development of bioinks with improved mechanical properties and biocompatibility will enable additive manufacturing such as 3D printing to make tremendous opportunities for regenerative medicine [127].

Instilling peristaltic activity to the tissue engineered intestine to establish gut motility will require correctly oriented smooth muscle cell regeneration and reinnervation. Advances have been made in technologies to achieve peristalsis through the combination of autologous smooth muscle cells and biomaterials to produce patch or tubular constructs [128]. Maintaining native smooth muscle organization appears to be critical to achieving functionally contracting smooth muscle [129]. Intact smooth muscle strips retained neural and glial markers and exhibited periodic contraction whereas smooth muscle cells cultured after enzymatic digestion did not. Innervation is not only an unmet need for bioengineering gastrointestinal tissue constructs. Enteric neuropathies such as achalasia, gastroparesis, intestinal pseudoobstruction, and chronic constipation are functional gastrointestinal disorders that result from primary and secondary forms of degenerative disease that affect the nerves and muscles in the gastrointestinal tract [130]. Mouse enteric neural crest cells transplanted into aganglionic gut spread along the endogenous myenteric plexus to form functionally integrated branching networks closely associated with endogenous neural glial networks, providing evidence for the use of enteric neural stem cell therapies [131]. The appendix, a vestigial organ, might provide a potential source of autologous neural stem cells for enteric neural cell therapy [132]. Geisbauer and colleagues investigated whether a mixture of enteric cells isolated from longitudinal and circular muscle of the gut could be used as a potential source of neural crest stem cells for cell therapy. When the isolated cells were mixed in collagen containing bFGF and injected into an aganglionic segment of jejunum, ganglion-like structures were generated with elongated synapses [133]. Innervation of tissue engineered constructs is fundamental to achieving or restoring gastrointestinal transit. Colon smooth muscle cells cultured in composite chitosan scaffolds were innervated by differentiated functional neurons derived from cocultured neural progenitor cells [134]. Likewise, human smooth muscle cells and neural progenitor cells were engineered into innervated sheets of smooth muscle and wrapped around tubular chitosan scaffolds [135]. After subcutaneous implantation, the construct became vascularized and the lumenal patency was maintained.

In addition to regulating peristalsis, the enteric nervous system in the intestine controls villi activity and the modulation of secretions from gut epithelial cells. Gut endocrine cells has an important role in regulating gastrointestinal activity by releasing serotonin, secretin, cholecystokinin, gastrin, and enteroglucagon and will be an essential component of the tissue engineered intestine. Nakase and colleagues investigated the regeneration of endocrine cells and the nerve system in a canine patch model of tissue engineered small intestine using a collagen sponge scaffold loaded with autologous gastric smooth muscle cells [136]. At 24 weeks after implantation of the scaffolds into the

middle of an isolated ileal loop, the location and number of endocrine cells staining positive for chromogranin A were almost identical to those of native mucosa. Nerve fibers were present in the regenerated smooth muscle layer and villi, but the myenteric plexus of Auerbach and the submucosal plexus of Meissner were not visible. The density of smooth muscle cells implanted into the scaffolds did not affect the thickness of the regenerated smooth muscle layer, which remained approximately half that of the native smooth muscle layer, indicating that other cues will be necessary to increase its thickness. The investigators suggested that the thickness of the muscle layer might be limited by the blood supply available to the regenerating tissue, which might be increased by the delivery of angiogenic factors from the scaffold. Grikscheit and colleagues also reported that ganglion cells were distributed in the locality of the Auerbach and Meissner's plexuses in tissue engineered small intestine [90].

Regeneration of the small intestine remains at an early stage of clinical development and has yet to provide a clear demonstration of improvement in nutrient absorption that will be valuable in a clinical therapeutic setting in humans. Although no models have unequivocally demonstrated functional neointestine with peristaltic activity, they indicate that it is feasible to engineer tubular segmental replacement of small bowel that incorporates innervated smooth muscle layers. Based on these findings, it may be possible to achieve incremental steps toward tissue engineering the intestine. For example, combining existing and established surgical procedures with the principles of tissue engineering and regenerative medicine may improve existing clinical outcome measures. An example of this type of approach was demonstrated by Nakao and colleagues, who showed that the longitudinal staples used during Bianchi's procedure could be replaced with SIS graft [137]. Refinement of other existing techniques could yield further advances toward viable options for tissue engineered intestinal constructs.

COLON

The colon is an important organ for water and sodium resorption and a storage pouch for waste products. Patients who undergo total colectomy are at risk for significant morbidities [138]. The surgical creation of an ileal pouch to create a reservoir provides only a limited solution and patients may still experience inflammation of the pouch (pouchitis), malabsorption, diarrhea, cramping abdominal pain, and fever [139].

Tissue engineering approaches similar to those used for the small intestine have been applied to the colon [140]. Consequently, many of the same challenges exist. Tissue engineered colon was achieved by seeding organoid units harvested from the sigmoid colon of neonatal Lewis rats, adult rats, and tissue-engineered colon itself onto a polymer scaffold that was implanted into the omentum of syngeneic adult Lewis rats. Tissue-engineered colon was generated from each of the donor tissue sources and the resulting architecture of the neocolon was similar to that of native tissue. When anastomosed to the native bowel, there was gross evidence of fluid absorption by the tissue engineered colon.

The choice of scaffold material used for colon tissue engineering will have an impact on the viability of the neocolon. Two of the main biological scaffold materials explored in gastrointestinal tissue engineering are SIS and chitosan. Relatively few studies have made direct comparisons of these materials in terms of their biocompatibility. Denost and colleagues looked at the in vitro and in vivo properties of two bioscaffolds composed of these materials [141]. No substantial difference was observed in vitro in terms of cell attachment and proliferation, but the chitosan hydrogel facilitated improved healing of a preclinical in vivo model of colonic wall defect, including regeneration of the smooth muscle layer.

A significant drawback reported with biological scaffolds such as chitosan is their weak mechanical properties. To enhance mechanical strength, Zakhem and Bitar reported using chitosan fibers circumferentially aligned around tubular chitosan scaffolds [142]. Tensile strength and strain, burst pressure, and Young's modulus were all increased in scaffolds that contained the fibers.

ANAL CANAL

Controlled storage and timely disposal of feces relies largely on the appropriate function of sphincter muscles that constrict the anal canal and maintain fecal continence. Fecal incontinence is a common disease, particularly in aging societies in which it has a huge impact on quality of life and incurs colossal health costs. Conservative estimates indicate that approximately 2% of community-dwelling adults experience regular fecal incontinence [143]. This figure increases to 50% in the institutionalized and geriatric population [144]. Conservative treatments for fecal

incontinence are ineffective in patients with more than mild symptoms and surgical interventions produce poor long-term benefit, with frequent complications [145].

Despite holding considerable promise, cell therapy for incontinence affecting the alimentary tract remains relatively unexplored in humans [146,147]. Although the technical feasibility of injecting autologous myoblasts for treating fecal incontinence in humans has been demonstrated, these studies have been unable to demonstrate integration of cells into the damaged sphincter or a direct improvement in the functional integrity of the sphincter muscle.

A fibrin-based bioengineered in vitro model of the internal anal sphincter (IAS) was described that demonstrated physiological functionality. This type of approach is likely to offer value in studying complex physiological mechanisms underlying sphincter malfunction [148,149]. The bioengineered sphincter was surgically implanted into the subcutaneous tissues of syngeneic mice and responded to the local delivery of bFGF, resulting in improved muscle viability, vascularization, and survival of the graft [150]. This field of research has continued to advance; the first study demonstrated the feasibility of transplanting bioengineered intrinsically innervated human IAS into mice. Isolated human IAS smooth muscle cells were cocultured with immortomouse fetal enteric neurons. The construct implanted into $RAG1^{-/-}$ mice became neovascularized and the physiological function of the myogenic and neuronal components was retained. The IAS construct exhibited characteristics of IAS physiology [151]. The feasibility of implanting a bioengineered sphincter construct consisting of human IAS smooth muscle cells was explored in the perianal region of athymic rats [152]. The constructs were well-tolerated and the recipients were able to produce stool normally. For this study, vascularization was increased by delivering platelet-derived growth factor. In addition to the anal sphincter, proof-of-concept studies have demonstrated that it is feasible to bioengineer autologous bioengineered innervated pylorus constructs that consist of circumferentially aligned smooth muscle cells that exhibit tonic contractile phenotype and basal tone [153].

However, the feasibility of scaling-up this type of approach from a rodent model to humans remains uncertain. Although it is technically possible to bioengineer rings of muscle in vitro on a scale comparable to that of human sphincter muscle [154], innervation, vascularization, and cell viability in larger constructs have yet to be tested in larger-sized preclinical models.

Regenerative medicine may also offer solutions to conditions in which existing medical and surgical procedures have failed. A condition in which this affects the alimentary tract is perianal fistulas that result from a connection between the anal canal and the perianal skin surface, creating an abnormal passageway for the discharge of pus, blood, and in some cases feces, resulting in significant morbidity. The goals of fistula treatment are eradication of perineal sepsis and fistula closure while posing a minimal risk for causing sphincter muscle damage. A difficulty in treating perianal fistulas is avoiding abscess formation caused by healing of the skin before closure of the tract. To address this, collagen anal fistula plugs have been devised to treat fistulas. Although early studies reported good healing rates with little or no risk to continence, long-term follow-up has revealed variable and disappointing success rates (24–78%) [155]. Reports of the plugs failing owing to dislodgment from the tracts indicate that this approach may not provide an ideal scaffold material to promote guided tissue regeneration and closure of the tract [155]. A possible solution to this problem is the use of scaffold materials that provide both optimal conditions for rapid cell infiltration when implanted into tissue cavities and mechanical strength to maintain an open scaffold structure [156].

IN VITRO MODELS

Improved understanding of gastrointestinal developmental biology opens up new opportunities for creating 3D tissue constructs that can be used to modeling disease, understand embryonic development, and provide construct sources for therapeutic applications [157]. Principles of regenerative medicine are increasingly being used to fabricate biomimetic models of the gastrointestinal tract. Challenges that exist with developing in vitro tissue models of gastrointestinal tissue include mimicking the 3D microenvironment, interactions among different cell types, and the microbiome. New technologies are being applied to address these, including using microfluidics to create channels lined by living cells in microengineered biomimetic systems that might offer new opportunities to replace conventional animal models in preclinical toxicology testing. This approach has been applied to a variety of organs including the intestine to provide organs-on-chips that exhibit physiological properties including peristalsis-like movement [158]. Dynamic culture in a defined perfusion bioreactor has also been reported to result in tissue models that are physiologically closer to native small intestine [159]. Microfluidic cell culture devices have been designed that contain villi- and crypt-like structures that resulted in epithelial cells tightly connecting to each other and displaying absorption and paracellular transport function [160]. The incorporation of physiological parameters also

appears to be important for establishing 3D in vitro tissue models of small intestine. Bioreactors used to culture decellularized segments of porcine jejunum have been used to coculture human Caco-2 cells with human microvascular endothelial cells. Compared with routine static Caco-2 assays, culture under dynamic conditions resulted in cell morphology that more closely resembled normal primary enterocytes [161]. Recapturing essential features of the cellular microenvironment is essential if in vitro tissue engineered models are to be used for functional studies such as cell growth, differentiation, absorption, or host–microbial interactions. The inclusion of native features such as accurately sized intestinal villi has been shown to facilitate cell differentiation along the villous axis [162]. Methods used to realize such structures include 3D natural and synthetic hydrogels created using a combination of laser ablation and sacrificial molding to achieve microscale structures that mimic the density and size of human intestinal villi [163]. The microbiome of the gastrointestinal tract is increasingly being recognized as a critical component to maintaining physiological homeostasis. Biomimetic in vitro intestinal models for investigating the adhesion and invasion profile of commensal and pathogenic organisms therefore have significant value in understanding microbe-induced intestinal disorders. To explore this interaction, synthetic 3D tissue scaffolds that support coculture of epithelial cell types have been used to provide microbial niches along the crypt–villus axis for modeling the interaction of a variety of commensal and pathogenic organisms [164].

CONCLUSION

The alimentary tract is a complex organ that is essential for maintaining physiological homeostasis. Tissue engineering and regenerative medicine for hollow visceral organs have been proposed as an approach for replacing damaged or diseased tissue, as demonstrated in humans with bladder [165] and airway tissue [15]. Although these "first-in-human" studies have rightly attracted much attention, there is a long way to go until a similar approach becomes routine in the relatively complex structures of the alimentary tract.

The past few decades have delivered a series of important studies that have used the innate ability of the alimentary tract to regenerate. Further studies are needed to demonstrate whether these approaches are transferable and of clinical value to humans. Fundamental challenges such as scalability will need to be addressed to enable the results obtained in small-animal models to progress into preclinical models applicable to humans. This will require refinement of the scaffolds used and the ability to seed limited quantities of cells available in an efficient manner onto the scaffolds. These are challenges that can be overcome and will allow regenerative medicine to progress in the alimentary tract in humans.

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CHAPTER

65

Extracorporeal Renal Replacement

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INTRODUCTION

The kidneys are vital organs that have critical excretory and regulatory roles in maintaining water and electrolyte homeostasis in the body while removing low−molecular weight (MW) toxins. Often overlooked, the kidneys also provide an essential endocrine function because they secrete important signaling molecules that interact with the cardiovascular, skeletal, and immune systems. The kidney was the first organ to be successfully transplanted and is one of the most common and effective transplantation surgeries performed in the world. However, global shortages of donor kidneys have proven to be a major barrier to treating kidney disease, leaving millions of chronic kidney disease patients without total functional replacement.

In the United States, 636,905 patients were listed as having end-stage renal disease (ESRD) at the end of 2012, as reported by the 2014 US Renal Data System (USRDS) database. According to the USRDS report, over 450,000 patients were receiving maintenance dialysis, 186,303 patients had a functioning kidney transplant, and 88,638 ESRD patients died during 2012 [1]. Adjusted rates of all-cause mortality are 6.1−7.8 times greater for dialysis patients than for individuals in the age-matched Medicare population [1]. The incidence rate of ESRD has plateaued and slightly decreased since 2009, with an adjusted incidence rate of 353 per million per year in 2012 [1]. The financial cost of ESRD is immense; spending for ESRD patients increased 3.2% to $28.6 billion in 2012, accounting for 5.6% of the Medicare budget [1]. A prior USRDS report estimated the cost of $54,900 per hemodialysis (HD) patient per year and $46,121 per peritoneal dialysis (PD) patient per year [2]. In contrast, transplant patients cost an average of $17,227 per patient per year [2]. The higher cost of maintenance dialysis compared with transplantation does not translate into better results; annual mortality for patients listed for transplant and awaiting a kidney is 6.3%, compared with only 3.8% for patients listed for transplant who received a kidney [2]. Although organ transplantation provides the best prognosis for survival, demand vastly outweighs the availability of donated organs. In 2014, the active waiting list was 2.8 times larger than the supply of donor kidneys [3].

Extracorporeal therapies to replace kidney function at least partially include HD, hemofiltration (HF), hemodiafiltration (HDF), and PD. These therapies replace the blood filtration function of the kidney by removing waste products and excess electrolytes via artificial or natural semipermeable membranes in extracorporeal circuits. These methodologies all address water and electrolyte balance as partial functional replacement of the kidney but are prone to various complications such as acidosis, volume overload, and uremic syndromes that accompany renal failure [4]. Conventional therapies fail to provide the lost endocrine functions of the kidney, and thus the metabolic, endocrine, and immunological roles of the functioning kidney are potential mechanisms for the difference in survival for patients who receive kidney transplants.

REQUIREMENTS OF A RENAL REPLACEMENT DEVICE

In the kidney, HF is accomplished by nephrons that generate ultrafiltrate (UF) from blood via glomeruli. Each glomerulus, a tuft of capillaries supported by a basement membrane and specialized epithelial cells called podocytes, is an efficient filter with a molecular weight cutoff (MWCO) of around 65 kDa. This allows for small molecules including small MW toxins such as urea to be removed from the bloodstream, whereas the cellular component of

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blood and larger proteins including albumin are retained. UF is passed into the renal proximal tubule portion of the nephron, a hollow tube of cells surrounded by capillaries, which receives the filtrate from the glomerulus and accomplishes the bulk of reclamation of salt, water, glucose, small proteins, amino acids, glutathione (GSH), and other substances. A renal replacement device thus must recapitulate the filtration function of the nephron. However, in addition to its role in water−electrolyte balance, the tubule also performs metabolic functions, including excretion of acid as ammonia and hydroxylation of 25-hydroxy-vitamin-D_3 among other metabolic and regulatory functions, which are not supplemented in conventional renal replacement therapies (RRT).

The kidney is responsible for the secretion of hormones that are critical in maintaining hemodynamics (renin, angiotensin II, prostaglandins, nitric oxide, endothelin, and bradykinin), red blood cell production via erythropoietin, and bone metabolism (1,25-$[OH]_2$−vitamin D_3 or calcitriol) [5]. Free-radical scavenging and GSH-metabolizing enzymes are synthesized by the kidney, which also provide gluconeogenic and ammoniagenic capabilities [6,7]. Catabolism of low -molecular weight proteins, including multiple peptide hormones, cytokines, and growth factors, is also accomplished by the kidney [8]. The kidney also has a less-recognized immunoregulatory function. Mammalian renal proximal tubule cells (RPTC) are immunologically active. RPTCs are antigen-presenting cells [9] that have costimulatory molecules [10] that synthesize and process a variety of inflammatory cytokines [10,11]. Long overlooked, the kidney is an important immunomodulatory organ. RRT has poor patient prognosis owing to the acute tubular necrosis (ATN) that results in the loss of the kidney's immunoregulatory function. Loss of immunoregulation results in a propensity to develop systemic inflammatory response syndrome (SIRS), sepsis, multiple organ dysfunction (MOD), and a high risk for death because of systemic immunologic or inflammatory imbalance. The endocrine and immunologic roles of kidney cells are also important in maintaining kidney and other vital organs' health under stress conditions [12] and are not provided by conventional RRT.

DEVICES USED IN CONVENTIONAL RENAL REPLACEMENT THERAPY

Water and solute homeostasis function of the kidney can be practically replaced by various dialysis or filtration methods including HD, HF, HDF, and PD. In these processes, synthetic membranes are used to remove water and solutes selectively based on diffusive or convective transport. In HD, in which solute removal from the blood is driven by a concentration gradient across a membrane, the process is dominated by diffusion. However, solute transfer also occurs to a lesser extent, convectively by a process of ultrafiltration, in which water and solutes move across the membrane [13]. This process is leveraged further in HF, in which pores in semipermeable membranes allow water and solutes across the membrane independent of the concentration gradient, and predominantly on the hydraulic pressure gradient across the membrane, which mimics the filtration function of the nephron. HDF combines HD and HF processes, in which both diffusion and hydraulic pressure are used as driving forces. In PD, similar to HD, solute removal is mainly driven by diffusion across a synthetic membrane; however, instead of solutes being removed directly from blood, solutes are removed from the peritoneal fluid of the patient, which avoids complications of blood access. PD has a higher safety profile, which enables dialysis treatments at home, overnight dialysis, and continuous ambulatory dialysis.

In HD, blood exits the patient's body through a catheter, which is delivered to a dialyzer via tubing and a blood pump (Fig. 65.1A). These dialyzers are commonly filled with hollow fiber membranes formed into a bundle. Within a hollow fiber dialyzer, blood flows through the lumen, the interior of each hollow fiber. Solutes in the blood interact with the fiber's semipermeable membrane, which allows for low-MW solutes such as urea and creatinine to pass through the porous fiber wall into the extracapillary space (ECS) of the dialyzer for removal, whereas blood cells and critical large proteins such as albumin and immunoglobulin are retained in the blood. Dialysate, an aqueous solution administered during dialysis, flows opposite the blood on the other side of the semipermeable membrane in the ECS. The dialysate has various concentrations of solutes that dictate the driving force to remove, retain, or add to solute concentrations in the blood. Movement of the solute is directed from high to low concentration so that toxins are removed and critical electrolytes in the blood are maintained.

In HF, blood is filtered using a pressure difference to drive water and solutes across a semipermeable membrane and into the filtrate compartment. Like dialyzers, hemofilters are routinely filled with hollow fibers. Positive pressure can be applied to the hemofilter's blood compartment (lumen side of the hemofilter) or a negative pressure can be applied to the filtrate compartment (ECS), creating a transmembrane pressure. No dialysate solution is used in HF; rather, the filtrate is collected and discarded and ultrapure replacement solution is administered to the patient to maintain water−solute balance, as shown compared with dialysis in Fig. 65.1B.

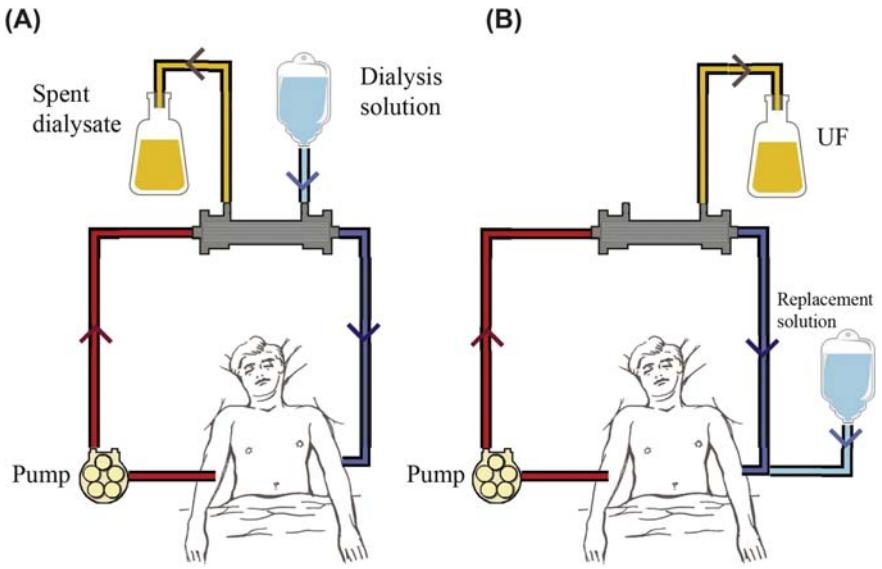

FIGURE 65.1 Diagrams of hemodialysis (A) and conventional venovenous hemofiltration (B) methods. *UF*, ultrafiltrate.

Membrane materials used in dialyzers and hemofilters are diverse, ranging from metals and ceramics to polymers. Critical to the choice of materials for dialysis membranes, biocompatibility with blood is a major concern, as are cost and manufacturing. Synthetic polymers have been the dominant membrane materials used for separation processes owing to combination of these factors. The most common polymers in the manufacturing of synthetic membranes are nondegradable ones, including cellulose derivatives such as cellulose acetate, nitrates, and esters, polysulfones, and their ether-containing derivatives polyethersulfones and polyarylethersulfone, polyacrylonitrile (PAN) derivatives, polyamides, polyimides, polyolefins such as polyethylene and polypropylene, polyvinylchloride, and fluorinated polymers such as polytetrafluoroethylene, and polyvinylidine fluoride.

ADVANCEMENTS IN CONVENTIONAL RENAL REPLACEMENT THERAPY DEVICES

Besides improvements in biocompatibility, synthetic membranes have been developed to better reproduce the physiologic process of glomerular ultrafiltration. In an attempt to recapitulate glomerular ultrafiltration and removal of "middle molecules," synthetic membranes with larger pore sizes and high water permeability have been developed. These "high-flux" membranes are commonly prepared with hydrophobic base materials, along with various hydrophilic components. These membranes can be mass produced, with wide ranges of hydraulic permeabilities for various treatments [14].

Pore size, therefore, is a critical issue in hemofilter design. Conventional polymeric membranes are typically cast or spun from polymers in solution, resulting in a heterogeneous dispersion of pore sizes and geometries within fibers or sheets, with ill-defined MWCO. New techniques using a nanoscale spinning process to control pore size, resulting in an increased uniformity of pores with desired values [15].

Based upon this technology, the main focus of recent membrane development has been to increase pore size while sharpening the MWCO of high-flux membranes to maximize removal of low MW proteins. This direction is based upon the idea that removal of a distinct class of uremic toxins, such as β2-microglobulin and myoglobin, while minimizing the loss of albumin could improve treatment outcomes of patients with ESRD. Superflux or protein-leaking membranes, provide greater clearances for low MW proteins and small protein-bound solutes, but with significantly higher loss of albumin than high-flux dialysis membranes. Other determinants of dialyzer performance may include: fiber bundle configuration, spacing, and sterilization techniques [16].

Membranes typically used today are purely artificial and are used in the form of hollow fiber modules, commonly based on cellulose or synthetic polymers, including polysulfone, PAN, and polyamide, used as such or modified with a variety of agents [17–19]. However, research in biomaterials and tissue engineering holds promise for significant future improvements. Improvements in membrane biocompatibility have reduced inflammatory reactions, but

off-the-shelf polymeric materials still induce a cascade of biochemical events leading to blood coagulation, thrombus formation, and often complement activation and inflammation. Systemic administration of anticoagulant such as heparin is required to solve these problems. However, the use of anticoagulants, may lead to additional complications such as hemorrhage, and thus researchers are trying to improve anticoagulation therapy by finding alternative antithrombotic agents such as hirudin [20] and methods of administration, such as the direct incorporation of bioactive agents onto the polymeric membrane itself [21], such as vitamin E or heparin [22].

Conventional membranes remove solutes only on the basis of molecular size, whereas the glomerulus also employs charge selectivity by the basement membrane. This nonselective nature of conventional membranes is another limitation to overcome. One possible solution to this problem is to grow cells on the hemofilter membrane to improve the selectivity of the membrane. Another approach is the design of "smart" membranes that have selective transport characteristics and contain a predetermined number and size of nonpassive nanoengineered pores that have specific interactions with solutes and solvents at the atomic level [23].

Despite improvements in membrane technologies allowing for more efficient dialysis, HF, or diafiltration therapies, all are suboptimal treatments for both acute kidney injury (AKI) and ESRD. These therapies do not meet the medical need to reduce mortality caused by AKI. Even in ESRD, the outcomes of patients receiving chronic dialysis therapy are still disappointing, with an annual mortality exceeding 25%, on average and a drastically shortened life expectancy of only 5 years [24]. Several groups have reported that the survival of critically ill patients with AKI could be improved by intensifying the dose of RRT [25–27], but the initial excitement generated by these reports has waned after studies demonstrated that the dialysis dose does not closely relate to outcomes [28–30].

RENAL ASSIST DEVICE: A MORE COMPLETE RENAL REPLACEMENT THERAPY

Complete RRT should replace metabolic and endocrine functions of the kidney not supplied by conventional RRT. One such approach, called the renal assist device (RAD), used living cells supported on synthetic scaffolds [31]. In this bioengineered device, nondegradable scaffolding materials were employed to provide physical support for renal cells grown within the device. The RAD was constructed using renal tubule progenitor cells [32,33] cultured on semipermeable polymeric hollow fiber membranes (polysulfone) on which an extracellular matrix was layered to enhance the attachment and growth of the epithelial cells [34] (Fig. 65.2A). These porous hollow-fiber synthetic membranes not only provide the architectural scaffold for these cells and allow for delivery of nutrients to support the cells, they provide immunoprotection during therapy, preventing potential immunologic reactions in patients or compromise of the cell device by the patient's immune system. With appropriate membranes, immunoprotection of cultured progenitor cells can be achieved concurrent with long-term functional performance as long as conditions support tubule cell viability [31].

In vitro experiments have tested transport and metabolic functions of the RAD using renal proximal tubule progenitor cells grown inside the lumen of hollow fiber devices with membrane surface areas of 97 cm^2 to 0.7 m^2, resulting in a device containing up to 10^8–10^9 cells [35]. The nonbiodegradability and pore size of the hollow fibers allowed the membranes to act as both scaffolds for the cells and an immunoprotective barrier. Confluent monolayer cells within the RAD exhibited morphological characteristics typical of differentiated tubule epithelia including apical microvilli, endocytic vesicles, and tight junctional complexes, as well as differentiated active transport properties, differentiated metabolic activities, and important endocrine processes [35]. RPTC cultured in the RAD were found to maintain their ability to synthesize and excrete ammonia, produce 1,25-$(OH)_2$–vitamin D_3 (the active form of vitamin D), and, through metabolic degradation, remove GSH from the perfusate. Taken together, these results suggested that the RAD had the ability to replicate the major differentiated transport, metabolic, and secretory functions performed by the healthy renal proximal tubule.

To assess the potential clinical translation of this technology, large-animal studies were undertaken using an RAD consisting of renal proximal tubule progenitor cells. In brief, dialysate from a conventional dialysis circuit can be passed into the RAD as a nutrient and oxygen source for the cells grown on the membrane. The membrane is both water- and solute-permeable, allowing for differentiated vectorial transport and metabolic and endocrine activity provided by the cells, and processed dialysate is discarded. Immunoprotection of cultured progenitor cells is achieved concurrent with long-term functional performance as long as conditions support tubule cell viability. In studies with acutely uremic dogs after bilateral nephrectomies, the RAD demonstrated replacement of filtration, transport, metabolic, and endocrine functions of the kidney [35–37]. Animals were treated with either an RAD or a sham control cartridge daily for either 7 or 9 h for 3 successive days or for 24 h continuously. Fluid and small solutes, including blood urea nitrogen (BUN), creatinine, and electrolytes, were adequately controlled in both groups, but

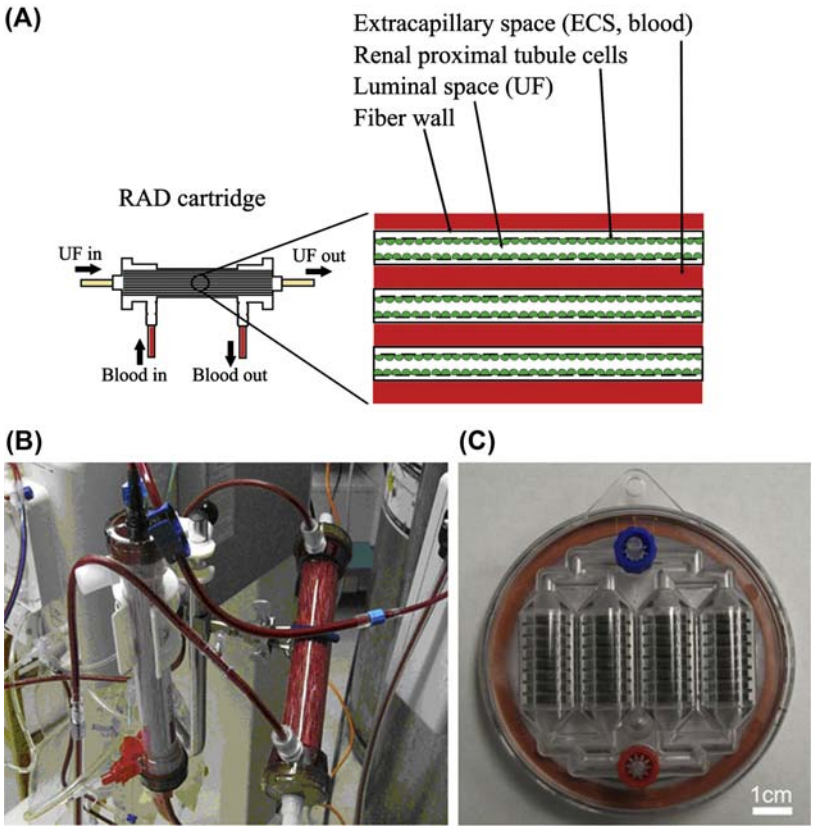

FIGURE 65.2 Depiction of renal assist device (RAD) (A), selective cytopheretic device (B), and bioartificial renal epithelial cell system (C) devices.

potassium and BUN levels were more easily controlled by RAD treatment. Furthermore, active reabsorption of K^+, HCO_3^-, and glucose and excretion of ammonia were accomplished only in RAD treatments. Glutathione reclamation from UF exceeded 50% in the RAD. Finally, uremic animals receiving cell therapy attained normal 1,25-$(OH)_2$—vitamin D_3 levels, whereas sham treatment resulted in a further decline from the already low plasma levels. Thus, these experiments clearly showed that the combination of an HF cartridge and an RAD in an extracorporeal circuit successfully replaced filtration, transport, and metabolic and endocrinological functions of the kidney in acutely uremic dogs [35—37].

Additional animal experiments in a porcine model investigated the impact of treatment with this device on the high mortality of sepsis complicated by AKI [38].

RENAL ASSIST DEVICE THERAPY OF ACUTE KIDNEY INJURY CAUSED BY SEPSIS

To examine the impact of cell therapy on the course of sepsis complicated by renal failure [38,39], a porcine model of septic shock was developed from the previous work [40,41]. Purpose-bred pigs were anesthetized and administered an intraperitoneal dose of bacteria, causing shock and renal failure. An hour later, continuous venovenous hemofiltration (CVVH) was initiated with either cell or sham RAD. Urine output and mean arterial pressure (MAP) declined within the first few hours after insult. Cell-treated animals survived 9.0 ± 0.83 h versus 5.1 ± 0.4 h ($P \leq .005$) for sham-treated animals. Serum cytokines were similar between the two groups, with the striking exception of interleukin (IL)-6 and interferon (IFN)-gamma. Treatment with the cell RAD resulted in significantly lower plasma levels of both IL-6 ($P \leq .04$) and INF-gamma ($P \leq .02$) throughout the experimental time course compared with sham RAD exposure. This controlled trial of cell therapy of renal failure in a realistic animal model of sepsis had several findings not immediately expected from a priori assumptions regarding renal function. Previously, although renal failure was strongly associated with a poor outcome in hospitalized patients, and chronic renal failure was associated with specific defects in humoral and cellular immunity, a direct immunomodulatory effect of

the kidney had not been accepted. In this trial, clear differences in survival and clear differences in a serum cytokine associated with mortality in sepsis were found between groups. The increased mortality in renal failure appears to be not attributable to inadequate solute clearance, but may arise from other bioactivity of the kidney.

Studies in both dogs and pigs have demonstrated that RAD treatment in a bioartificial kidney circuit improves cardiovascular performance associated with changes in cytokine profiles and confers a significant survival advantage in septic or endotoxin animal models [38,39,42].

CLINICAL EXPERIENCE WITH A RENAL ASSIST DEVICE TO TREAT ACUTE KIDNEY INJURY

Encouraging preclinical data led to US Food and Drug Administration (FDA) approval for an investigational new drug and Phase I/II clinical trials. The first human clinical study of the bioartificial kidney containing human cells was carried out in 10 intensive care unit (ICU) patients with AKI receiving CVVH [43]. This study demonstrated that the RAD can be used safely for up to 24 h. Cardiovascular stability was maintained and increased native renal function, as determined by elevated urine outputs, was temporally correlated with RAD treatment. All patients were critically ill with AKI and multiple organ failure (MOF), with predicted hospital mortality rates between 80% and 95%. Six of the ten treated patients survived past 30 days, with mortality reduced to 40%. The human renal tubule cells contained in the RAD demonstrated differentiated metabolic and endocrinologic activity in this ex vivo situation, including GSH degradation and endocrinologic conversion of 25-OH−vitamin D_3 to 1,25-$(OH)_2$−vitamin D_3. Plasma cytokine levels suggest that RAD therapy produces dynamic and individualized responses in patients, depending on their unique pathophysiologic conditions. For the subset of patients who had excessive proinflammatory levels, RAD treatment resulted in significant declines in granulocyte-colony stimulating factor (G-CSF), IL-6, IL-10, and especially the IL-6/IL-10 ratio, suggesting a greater decline in IL-6 relative to IL-10 levels and a less proinflammatory state. These favorable Phase I/II trial results led to a randomized, controlled, open-label Phase II trial conducted at 12 clinical sites in the United States [30]. A total of 58 patients with ARF requiring CVVH in the ICU were randomized (2:1) to receive CVVH plus RAD (n = 40) or CVVH alone (n = 18). Despite the critical nature and life-threatening illnesses of the patients enrolled in this study, the addition of the RAD to CVVH resulted in a substantial clinical impact on survival compared with the conventional CVVH treatment group. RAD treatment for up to 72 h promoted a statistically significant survival advantage over 180 d of follow-up in ICU patients with AKI and demonstrated an acceptable safety profile. Cox proportional hazards models suggested that the risk for death was approximately 50% of that observed in the CVVH-alone group.

A follow-up Phase IIb study to evaluate a commercial manufacturing process was not completed owing to difficulties with the manufacturing process and clinical study design, which identified two key missing components for successful commercialization of cell-based therapy: (1) a reliable cell source to manufacture thousands of cell devices, and (2) a cost-effective storage and distribution process for cell devices.

IMMUNOMODULATORY EFFECT OF THE RENAL ASSIST DEVICE

As described earlier, RAD treatment altered systemic circulating cytokine levels in animal and human experiments. In endotoxin-challenged and gram-negative peritonitis uremic dog models, plasma levels of IL-10 were significantly higher in RAD-treated animals [38,39]. The role of IL-10 in regulating immune response continues to be elucidated, but data suggest that IL-10 levels influence outcome in endotoxin shock and gram-negative sepsis. Several reports have demonstrated that administration of recombinant IL-10 is protective against gram-negative septic shock in murine sepsis models [44,45]. Another study in a similar model demonstrated that administration of antibodies to IL-10 was associated with higher mortality [46]. The mechanism underlying the link between proximal tubule function and IL-10 levels remains to be detailed, but preliminary data suggest that renal production of IL-6 induces liver production of IL-10 [47]. In gram-negative septic pigs without nephrectomy, RAD treatment significantly reduced plasma circulating levels of IL-6 and INF-gamma [42]. The difference in IL-6 concentrations is especially noteworthy because the plasma elevations of this proinflammatory cytokine have been directly correlated to outcome in patients with SIRS [48]. The lower concentration of plasma INF-gamma may be important owing to its central role in the inflammatory response. INF-gamma stimulates B-cell antibody production, enhances polymorpholeukocyte phagocytosis, and activates monocytes and macrophages to release proinflammatory cytokines

[49–51]. Excessive rates of INF-gamma production by natural killer cells have correlated with progression to lethal endotoxin shock in mice [52]. Further support for an immunomodulatory role of renal tubule cells has been suggested in the Phase I/II clinical trial of the RAD containing human renal tubule cells [53]. Patients treated in this study had a wide spectrum of plasma cytokine levels. The subset of patients who presented with very high plasma cytokine levels and who were treated for an adequate period showed that RAD treatment resulted in significant reductions in G-CSF, IL-6, and IL-10 levels. The greater relative reduction in the IL-6/IL-10 ratio suggests that renal tubule cell therapy may rebalance the excessive proinflammatory response with the concurrent antiinflammatory response. These results are consistent with an immunomodulatory role for the RAD in patients with ATN and MOF. To evaluate the RAD's influence further on local inflammation in tissue and distant organ dysfunction, especially in the lungs, a study compared bronchoalveolar lavage (BAL) fluid from cell-RAD–treated and noncell, sham-treated groups in a pig model with septic shock with AKI [54]. The levels of total protein in BAL were significantly higher in sham control animals than in the RAD group (143 ± 11 compared with 78 ± 10 μg/mL, respectively; $P > .05$). Proinflammatory cytokines, including IL-6 and IL-8, were markedly elevated in the control group. These results demonstrate an important role for renal epithelial cells (RECs) in ameliorating multiorgan injury in sepsis by influencing microvascular injury and the local proinflammatory response. A more promising direction to improve the outcome of AKI is to better understand and interrupt the pathophysiologic processes that are activated in AKI, resulting in distant MOD and eventually death. AKI results in a profound inflammatory response state resulting in microvascular dysfunction in distant organs [55,56]. Leukocyte activation has a central role in these acute inflammatory states. Disruption of the activation process of circulating leukocytes may limit microvascular damage and MOD [57]. The RAD appears to influence systemic leukocyte activation and the balance of inflammatory cytokines and may alter the proinflammatory state of AKI and ultimately improve morbidity and mortality.

Our group has developed a novel synthetic membrane embedded in an extracorporeal device to bind and inhibit circulating leukocytes. This selective cytopheretic device (SCD) is an acellular device that mimics many of the immunomodulatory aspects of the RAD. The SCD improved septic shock survival times in preclinical animal models [58] and improved the survival outcome of ICU patients with MOF in a prospective, single-arm, single-center study [59], a prospective, single-arm, multicenter US study [60], and a randomized, multicenter clinical trial in which SCD-therapy patients were maintained within recommended ionized calcium (riCa) range [61].

SELECTIVE CYTOPHERETIC DEVICE

SCD therapy is an acellular device-based, extracorporeal blood treatment in which a patient's blood is passed through a hemofilter followed by an SCD setup in series (Fig. 65.2B) while regional citrate anticoagulation is administered. The proprietary circuit [62] was designed to combine small solute clearance by the HF portion of the circuit, whereas the SCD portion provides immunomodulation via a low–shear stress (SS) environment for blood cells passing by the fibers within the SCD. SS encountered by blood cells within a hemofilter is greater than arterial SS in the body (>30 dyn/cm^2), whereas SS encountered within the SCD approximates capillary SS (<1 dyn/cm^2), which provides an environment for leukocyte adherence [63]. The SCD was evaluated in a porcine model of *Escherichia coli*–induced septic shock and demonstrated increased survival time for SCD-treated groups [64], along with an ability to lower neutrophil activity (serum myeloperoxidase and CD11b cell-surface expression) and diminish neutrophil tissue invasion [58].

In several clinical trials for the treatment of patients with AKI and MOD, SCD treatment affected multiple leukocyte activation parameters, lowered proinflammatory cytokines, and elevated antiinflammatory cytokines [59,60,65]. In a later Phase I/II clinical trial for the treatment of AKI, 19 patients received SCD therapy (CVVH plus SCD) and maintained a postfilter ionized calcium level in the protocol's recommended riCa range (≤0.4 mmol/L) for 90% or more of the therapy; this resulted in a 60-day mortality of 16% (3 of 19) compared with control subjects (CVVH alone) and a mortality of 41% (11 of 27) ($P = .07$) [61]. Furthermore, for patients treated with SCD within riCa, dialysis dependence was significantly lower: 0% (none of 16) compared with control patients: 25% (4 of 16) ($P = .03$) [61]. Ongoing studies are evaluating dose–response effects and full elucidation of the mechanism of action of this device.

The SCD may provide a novel approach to the immune dysregulation in ESRD. This approach may be further tested in clinical studies as an innovative intervention to ameliorate the chronic proinflammatory state of ESRD and retard accelerated atherogenesis associated with chronic renal failure [66]. A pilot trial was undertaken to explore the impact of SCD therapy in ESRD patients, with 15 ESRD patients receiving HD with C-reactive protein (CRP) levels greater than 5 mg/dL. An excellent safety profile was observed for SCD therapy with no decline in

leukocyte or platelet counts. Monocyte (MO) phenotypes in these patients was determined for peripheral blood MO using flow cytometry. SCD therapy promoted a shift in MO phenotype from predominantly CD14 expressing MO at baseline or before SCD therapy to CD14 expressing MO after SCD therapy [67]. A significant shift in MO population phenotype afforded by a single SCD therapy session was observed ($P < .013$) [67]. In a subset of patients ($n = 7$) presenting with type 2 diabetes mellitus (T2D), this persistent decline in MO CD14 expression was sustained for as long as 2 weeks after therapy [67]. These results demonstrate that SCD therapy has the potential to modulate the chronic proinflammatory state in ESRD patients.

Following up on the finding in ESRD patients with T2D, SCD therapy was evaluated in an Ossabaw miniature swine model of insulin resistance with metabolic syndrome. Treatment with the SCD in this porcine model demonstrated a decline in circulating neutrophil activation parameters and MO counts [68]. These changes were associated with improvements in insulin resistance as determined with intravenous glucose tolerance testing [68]. Improvements were also reflected in the lowering of homeostatic model assessment insulin-resistant scores for up to 2 weeks after SCD therapy [68]. These promising findings allow for the planning of clinical studies for new indications, including treatment of obese T2D patients not presenting with ESRD. Other potential indications for SCD treatment may include other disease states associated with acute or chronic inflammation, including patients receiving cardiopulmonary bypass [69].

CHALLENGE OF CELL-BASED DEVICE: ROBUST CELL SOURCE

To continue to pursue a cell-based device and move past manufacturing hurdles that hindered RAD development, a robust cell supply needed to be developed although human donor tissue is limited; focus was directed toward improving renal cell yield per human kidney. An enhanced propagation (EP) method to expand renal epithelial progenitor cells from available adult human kidney transplant discards was successfully developed and implemented to provide the biomass necessary for renal cell-based therapeutics [70]. The EP method was successful in generating renal progenitor cells using kidneys from a wide range of donors, including suboptimal health profiles consisting of long-term diabetes, hypertension, age greater than 70 years, donation after cardiac death with prolonged warm ischemia, and one donor with ESRD receiving dialysis for over 2 years. Under EP, cell yields are consistently higher than 10^{11} cells/g cortex enabling the manufacture of over 100,000 devices per donor kidney for use in acute disease indications. In addition, greater than 1.8×10^{10} progenitor cells/g cortex were isolated from the one ESRD kidney tested, which strongly supports the hypothesis that autologous therapy may be possible using cells derived from a renal sample taken early in the course of chronic renal failure. In this regard, a conservative 0.1-g renal cortex sample excised from an ESRD patient would allow the fabrication of at least 18 devices containing up to 10^8 cells/unit. Cells isolated under EP method compared favorably with respect to metabolic function and morphologic phenotype with cells used in the RAD therapy clinical trials. Cryopreservation of the EP-isolated REC, which is necessary to allow for processing of these large cell yields, has been advanced using FDA-compliant procedures. Cell death postthaw has been minimized, with therapeutic potential of progenitor-derived REC conserved as evaluated using a surrogate efficacy panel consisting of lipopolysaccharide-stimulated IL8 secretion and γGT enzyme activity.

CHALLENGE: COST-EFFECTIVE STORAGE AND DISTRIBUTION FOR CELL DEVICES, BIOARTIFICIAL RENAL EPITHELIAL CELL SYSTEM DESIGN

A major obstacle in the widespread adoption of renal cell therapy to augment RRT is the lack of an "off-the-shelf," readily available system for on-demand use. Distribution, storage, and preparation for therapeutic use at point-of-care facilities have long stood as the major impediments to tissue engineered therapies [71]. The bioartificial renal epithelial cell system (BRECS), shown in Fig. 65.2C, is the first all-in-one cell culture vessel that can be cryopreserved and function as a cryostorage device and a cell therapy delivery system [72] to overcome this critical barrier to tissue engineered products' clinical translation [71].

The BRECS was designed to achieve the support of a much greater density of cells in a much lower fill volume of the previous RAD design [72], along with the capacity to cryopreserve the full unit to facilitate distribution. The polysulfone hollow fibers used as the scaffold in the previous RAD design were limited in the cell attachment surface area and were prone to fracture during cryopreservation and thaw processes, a critical element for storage and

distribution of a cell therapy device. The cell-seeding scaffold chosen for the BRECS, niobium-coated carbon disks (Cytomatrix, Woburn, MA), was selected based on its biologically inert, nonbiodegradable, and favorable thermomechanical properties for cryopreservation. Cell culture media containing oxygen and nutrients were perfused through and around the porous disks housed within the BRECS. Growth of adequate cell numbers to achieve a therapeutic impact was possible owing to the disks' high surface area. High porosity (80–90%) allowed for sufficient, homogeneous delivery of oxygen and nutrients to the cells on the disks through a balance of convective and diffusive flows across and through the scaffold. The other BRECS components, including the polycarbonate housing and gasket materials, were carefully selected and thoroughly tested to withstand cryogenic temperatures while maintaining an uncompromised sterile internal BRECS environment.

Many different BRECS prototypes were evaluated during its development phase. In brief, prototypes were manufactured using computer numeric code machining, which allowed for initial evaluation of the combined perfusion culture and cell therapy platform. This device demonstrated uniform flow distribution during in vitro perfusion and all device components remained intact, with no indication of degradation over 6 months. Further advancements to BRECS technology had progressed through BRECS prototypes made by stereolithography toward an injection-molded design that can be mass produced [73].

In vitro cell viability and metabolic activity were confirmed in the BRECS by measuring lactate production and oxygen consumption [72]. Both metrics of metabolism were consistent throughout the duration of perfusion culture, with an interpolated total cell number of 2×10^8 cells. The average oxygen consumption rate was similar to previously reported values for metabolically active cells [31]. To determine whether RECs in BRECS maintained renal differentiated phenotypic characteristics over time in perfusion culture, human REC-seeded disks from BRECS units were processed for immunohistochemical analysis of selected renal cell differentiated markers acetylated tubulin (AT-1) and zona occludens (ZO-1). AT-1, a marker for apical central cilia of proximal tubule cells, exhibited regular staining in central regions of cells grown on disks. ZO-1, a marker for epithelial tight junctions, displayed strong expression along the surface of the cells. ZO-1–positive tight junctions and punctate AT-1–positive central cilia indicate polarized epithelium and were evident in all disks tested. Renal cell-specific catabolic function of the BRECS was confirmed by assessing GSH degradation in supplemented perfusion media [72]. GSH degradation rates in BRECS remained stable over 90-day studies, indicating long-term, sustained differentiated renal cell function in the device.

Using a cryopreservation protocol employing commercially available cryopreparative solutions (HTS-Purge, Biolife Solutions, Bothell, WA) and crypreservation (CryoStor 10, Biolife Solutions, Bothell, WA), BRECS were cryopreserved with high average cell retention and high average cell viability for human REC-seeded disks [72]. Cells after cryopreservation were shown to maintain renal cell-specific metabolic functions and retain renal cell-differentiated markers AT-1, and ZO-1, which suggested that the BRECS should provide renal cell-specific metabolic supplementation when used in conjunction with conventional RRT.

These data demonstrate that the BRECS is the first single device that can serve as a culture vessel to maintain cells, can reach cryopreservation temperatures as a full unit, and finally, can be reconstituted to provide cell therapy [72,73]. Having this storage capacity makes both emergent and acute use feasible.

Functionally, the BRECS replicates many of the metabolic replacement functions of the kidney in a way similar to that of the previous RAD. However, because of differences in materials and design, the BRECS does not replicate the molecular transport control of the RAD.

BIOARTIFICIAL RENAL EPITHELIAL CELL SYSTEM AS AN EXTRACORPOREAL THERAPY TO TREAT ACUTE KIDNEY INJURY

Following the same clinical translation path as the RAD, the BRECS was then tested in large-animal models to determine the safety profile and potential efficacy. In an acute septic shock–associated MOD porcine model, intraperitoneal administration of high-dose *E. coli* resulted in a decline in arterial blood pressure along with a reduction in vital organ perfusion. Without intervention, acellular control animal survival time was 7.6 ± 0.5 h [74]. Animals receiving BRECS therapy surviving through the predetermined study end point of 16 h were assigned a survival time value of 16 h. Average survival time for the BRECS-treated group was 13.0 ± 0.9 h ($P < .001$), which was significantly extended compared with the acellular cohort [74]. Furthermore, MAP and cardiac output (CO) declined over the septic time course for both acellular and BRECS treated groups. However, both MAP and CO were better maintained in BRECS-treated animals [74]. Hematocrit measurements demonstrated stabilization of capillary leak in BRECS-treated group whereas hematocrit increased rapidly in acellular controls. Studies show that no adverse

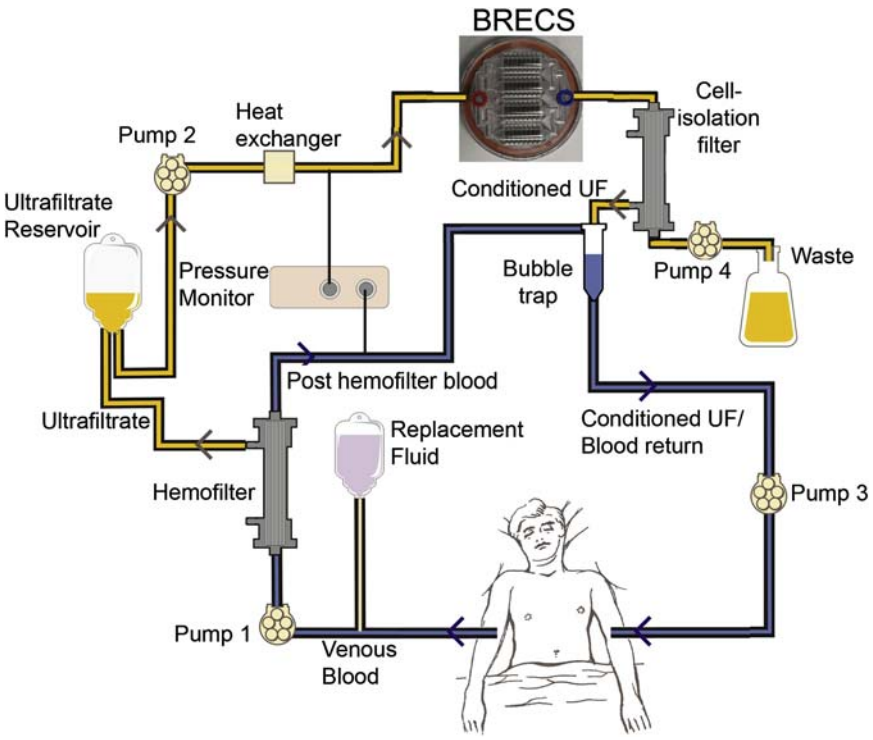

FIGURE 65.3 Diagram of bioartificial renal epithelial cell system (BRECS) extracorporeal circuit. *UF*, ultrafiltrate.

reactions attributable to the BRECS have been recorded. A schematic of a proposed BRECS therapy circuit for potential human clinical trials is depicted in Fig. 65.3.

WEARABLE BIOARTIFICIAL KIDNEY IN PRECLINICAL END-STAGE RENAL DISEASE MODEL

The development of cell therapy for treating chronic kidney disease such as ESRD has a number of technical challenges including the critical requirement of durable cell therapy and device longevity. One approach being developed is a wearable bioartificial kidney (WeBAK), which uses sorbent technology to regenerate peritoneal dialysate, which is perfused through the BRECS, to maintain cells within the cell therapy device. Similar to using UF to sustain RAD and BRECS in circuits with the patient's blood to treat AKI, dialysate is used as a nutrient and oxygen source for cells in the BRECS for the WeBAK. A large-animal ovine model was developed to evaluate BRECS supported by PD. Anephric sheep received continuous-flow PD in an extracorporeal circuit including the BRECS or acellular sham devices for up to 7 days, during which metabolic, immunologic, and endocrinologic parameters were evaluated [75]. Uremic control was achieved using continuous-flow PD. Cell viability and metabolic activity were maintained over the duration of treatment via the extracorporeal peritoneal fluid environment [75].

Although the WeBAK has not yet been evaluated in clinical studies for long-term use in ESRD, renal cell therapy paired with conventional RRT delivered from an extracorporeal circuit holds promise as a developing technology. A clinical trial failed to show survival benefit from increased doses of HD above standard care for uremic control [76], confirming that no benefit is likely to be achieved through conventional dialysis approaches to treat ESRD. Patients with ESRD display elevated levels of CRP and proinflammatory cytokines including IL-1, IL-6, and tumor necrosis factor [77–79]. All of these parameters are associated with enhanced mortality in ESRD patients. Specifically, IL-6 has been identified as a single predictive factor closely correlated with mortality in HD patients [77]. Although all ESRD patients could conceivably benefit from a bioartificial kidney, patients in the inflammatory stage who display elevated levels of chronic inflammation markers (such as IL-6 and CRP) would likely benefit most. Clinical translation will address long-term cell durability, biocompatibility, and administration protocols and additional accessory equipment required for bioartificial kidney therapy.

COMPLETE BIOARTIFICIAL KIDNEY SYSTEM FOR USE IN END-STAGE RENAL DISEASE

For the ESRD patient population, there are obvious limitations to using an extracorporeal RAD connected to an HF circuit. Ideally, a bioartificial kidney suitable for long-term use in ESRD patients would be capable of performing continuously, like the native kidney, to reduce risks from fluctuations in volume status, electrolytes, and solute concentrations and maintain acid−base and uremic toxin regulation. Such treatment requires the design and manufacture of a compact implantable or wearable dialysis apparatus and the development of miniaturized renal tubule cell devices with long service lifetimes.

Attempts have been made to develop wearable dialysis systems to improve the portability of RRT. Gura et al. published research regarding a lightweight wearable, continuous ambulatory ultrafiltration device consisting of a hollow fiber hemofilter, a battery-operated pulsatile pump, and two micropumps to control heparin administration and ultrafiltration [80]. This device regenerates dialysate with activated carbon, immobilized urease, zirconium hydroxide, and zirconium phosphate, similar to the once commercially available REDY dialysis system. Ronco and Fecondini described a wearable continuous PD system consisting of a double-lumen dialysate line with a peritoneal catheter, a miniaturized rotary pump, a circuit for dialysate regeneration, and a handheld computer as a remote control [81]. These systems still rely on inconvenient dialysate and expensive dialysis regeneration devices and/or dialyzers, but they promise to improve the convenience of dialysis.

In contrast to wearable dialysis systems, a hybrid bioartificial kidney integrates tubule cell and filtration functions. The first bioartificial kidney, consisting of a passive hemofilter and an active renal tubule cell bioreactor, has consistently demonstrated excellent safety and effectiveness in animal studies and FDA-approved human clinical trials, as described previously [16,30,42,53]. A major drawback of the current version of the bioartificial kidney is its large size, owing to the requisite extracorporeal circuit with peristaltic pumps to provide driving pressure for HF.

A new smaller and more durable RAD is being developed by Humes and colleagues. In collaboration, Fissell and colleagues are developing a nanopore membrane [82] to replace the filtration function of the glomerulus without the hemofilters and mechanical pumps of existing dialysis machines. A filtration device based on nanopore membrane technology would be implantable [83]. Further refinement of the RAD would be encouraging for ESRD patients because in principle, such a tissue-engineered device could be free of dialysate or replacement fluid while providing functions of healthy kidney that are not offered by current dialysis strategies. The combination of cell therapy and solute clearance could be a viable RRT, conferring dialysis independence to the patient.

FUTURE ADVANCEMENTS FOR WEARABLE AND AMBULATORY RENAL REPLACEMENT THERAPIES

Critical to the success of wearable and ambulatory RRT, including a wearable artificial kidney, a number of technical hurdles remain to be overcome, including creating a miniaturized, automated, safe dialysis platform to enable portability. Complex systems including multiple sensor feedback with real-time response capability would be required for combined cell therapy to augment conventional uremic toxin clearance and ensure proper cell maintenance within the cell therapy device and safe water−electrolyte balance for the patient concurrently. Real-time sensor technologies such as esophageal Doppler monitoring and peripheral waveform analysis may provide the basis for measures of central hemodynamic parameters critical to therapy. Likewise, bioimpedance and hematocrit monitoring may provide the basis for measuring changes in blood volume, because various techniques have been deployed in outpatient maintenance HD [84]. Chemical-field effect transistors offer the possibility of measuring electrolyte levels in a protein-free UF and reading out the potassium level to patients, who could then alter their diet or treat themselves with potassium-absorbing resins. Flow probes to determine the flow rate within the extracorporeal circuit and pressure sensors with safety features will be required for clinical implementation. Similar to portable or ambulatory PD systems described in literature, a WeBAK system would likely require a central processing and control unit that would receive information from sensors and determine actions for clinical management and control of the therapeutic circuit.

Other important steps toward a fully functional WeBAK include miniaturization of critical components such as pumps and power supplies or batteries. Each component of the completed system that requires power would have to be supplied by high−energy density battery packs. Technological advances in batteries and efficiency of electrical components such as pump motors and central processing units should enable a wearable dialysis system with cell therapy in the relatively near future. Although no wearable dialysis system exists, advancements in pump

miniaturization and technology to reduce the volume of regenerated dialysate during continuous portable PD are important steps to an engineered solution [85–88].

With regard to the biological component of a WeBAK, the durability of cell-based therapy suggests that the cell device itself could be maintained for at least 6 months under ideal temperature and nutrient conditions in a chronic use application without the need for replacement, which is ideal for treating ESRD with a continuous PD circuit [75].

CONCLUSION

Despite all of the advances in extracorporeal RRT, a portable, continuous, dialysate-free artificial kidney device has not yet been developed, which remains the ultimate goal of this technological progress. The enabling platform technologies discussed in this review can be seen as steps toward this overall goal from theoretical designs to prototypes evaluated on the laboratory bench, and to medical products used at the bedside. Future research requires an integrative, multidisciplinary approach to enable technologies to be brought together and tested in preparation for clinical translation. High priority must be given to sensing and regulating extracellular fluid volume, with feedback mechanisms to adjust ultrafiltration and reabsorption by the bioartificial kidney. Also, patient safety must be ensured when monitoring other critical aspects of therapy and fail-safe mechanisms to avoid acidosis or uremic syndromes.

The critical building blocks of an autonomous bioartificial kidney are advancing rapidly; revolutionary clinical trials are under way at multiple medical centers. The technologies that will enable advances to a more autonomous, dialysate-free system are under development. In addition, progress has been made in the field of cryopreservation, and thus the ability to manufacture, store, and distribute bioartificial organs is advancing. We will likely see quantum advances in renal replacement in the very near future.

List of Acronyms and Abbreviations

AKI Acute kidney injury
ARF Acute renal failure
AT-1 Acetylated tubulin-1
ATN Acute tubular necrosis
BAL Bronchoalveolar lavage
BRECS Bioartificial renal epithelial cell system
BUN Blood urea nitrogen
CA Cellulose acetate
CO Cardiac output
CRP C-reactive protein
CVVH Continuous venovenous hemofiltration
ECS Extracapillary space
EP Enhanced propagation
ESRD End-stage renal disease
FDA U.S. Food and Drug Administration
G-CSF Granulocyte-colony stimulating factor
GSH Glutathione
HD Hemodialysis
HDF Hemodiafiltration
HF Hemofiltration
ICU Intensive care unit
IFN Interferon
IL Interleukin
MAP Mean arterial pressure
MO Monocyte
MOF Multiple organ failure
MW Molecular weight
MWCO Molecular weight cut off
PAN Polyacrylonitrile
PD Peritoneal dialysis
PTFE Polytetrafluoroethylene
RAD Renal assist device
REC Renal epithelial cell
riCa Recommended ionized calcium

RPTC Renal proximal tubule cell
RRT Renal replacement therapy
SCD Selective cytopheretic device
SIRS Systemic inflammatory response syndrome
SS Shear stress
T2D Type 2 diabetes
UF Ultrafiltrate
USRDS United States renal data system
WeBAK Wearable bioartificial kidney
ZO-1 Zona occludens-1

Glossary

Hemodialysis membrane A synthetic membrane used in dialysis that is employed to separate toxins from the blood through a diffusion-dominated process.
Hemofiltration membrane A synthetic membrane used in filtration of the blood to remove toxins in a process dominated by hydraulic pressure.
High-flux membrane A membrane used in hemofiltration with larger pores and higher hydraulic conductivity than conventional membranes, which allows for the removal of higher−molecular weight toxins.
Synthetic membrane A fabricated structure that enables the separation of mixtures. Conventional membranes accomplish this separation based on molecular size, because lower−molecular weight species are able to pass through pores in the membrane.

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CHAPTER

66

Regenerative Medicine Approaches for the Kidney

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INTRODUCTION

Kidney disease is a leading cause of death globally [1]. Among its most common forms are acute kidney injury (AKI), chronic kidney disease (CKD), and end-stage renal disease (ESRD). In AKI and CKD, the kidney loses its capacity to function and filter blood efficiently. Furthermore, AKI can often deteriorate into CKD, which affects 8–16% of the global population [2]. Medical therapies for kidney diseases such as AKI and CKD primarily involve hemodialysis or kidney transplantation. Dialysis can replace renal filtration of metabolic waste from blood, but it serves as a supportive treatment that manages symptoms and delays disease progression. In addition, dialysis cannot replace other critical kidney functions, such as synthesis of erythropoietin (EPO), which stimulates red blood cell production [3]. Consequently, treatment of renal disease should promote efficient regeneration of functional renal-specific cells or stem cells; thus, a cell-based approach that replaces or restores damaged renal cells would be a promising alternative to current treatment options.

AKI and CKD can progress to ESRD, a severe condition affecting multiple organ systems. Kidney transplantation provides the only definitive treatment for ESRD; however, the supply of transplantable donor kidneys meets less than 20% of the demand [4]. To address this issue, tissue engineering (TE) and regenerative medicine (RM) approaches have been suggested as promising solutions. Stem cell biology and cell culture technologies have enabled researchers to identify and characterize kidney-derived cells and various types of cells that could be used to treat renal diseases [5,6]. The simplest approach uses cell transplantation, in which single therapeutic cells can be administered to a patient with kidney failure to restore renal function. Because this approach is easier to administer and less invasive than other alternatives, most studies have been performed using cell therapy strategies [7,8].

Whereas various cells have been studied for potential use in cell therapy [5], engineering of renal constructs has also been attempted to facilitate renal regeneration [9,10]. In this approach, the engineered constructs contain extracellular matrix (ECM), soluble factors, and a scaffolding system to guide in vitro cultured cells. The scaffold provides support to form three-dimensional (3D) renal structures that will lead to efficient integration with the host after implantation. This approach is ideally applicable to more serious renal conditions that require replacement of diseased kidney tissues.

Compared with these two cell-based approaches, a strategy that uses endogenous cell sources is emerging as a promising alternative for renal regeneration [11]. This technique, known as in situ tissue regeneration, takes advantage of the body's natural healing processes and the ability to recruit endogenous kidney cells for kidney regeneration. For efficient renal regeneration in situ, there are two goals: (1) redesign of damaged renal tissue after injury toward appropriate microenvironments, and (2) effective infiltration of host cells containing therapeutic potential into the recreated renal niche.

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In this chapter, cell sources that are used for cell-based therapies and associated applications to treat kidney diseases will be reviewed. In addition, several approaches to engineering renal constructs and in situ kidney regeneration will be presented.

CELL-BASED THERAPY

Cell transplantation and implantation of engineered kidney constructs have been considered as promising therapeutic methods. For both applications, establishment of a refined cell culture system is essential to obtain a sufficient number of therapeutic cells for later transplantation. To this end, various types of cell sources have been identified, characterized, and used to treat renal diseases (Table 66.1).

Cell Sources: Kidney Tissue–Derived Stem and Primary Cells

The adult kidney contains over 20 heterogeneous types of cells distributed among the various compartments, including vascular, interstitial, glomerular, and tubular structures with architectural complexity [12]. Through a

TABLE 66.1 Application of Cell Therapy for Kidney Regeneration

Cell Types and Origin	Cell Subtypes	In Vitro or In Vivo Animal Model	References
Renal progenitor cells from the Bowman capsules	Human $CD133^+$ cells from the Bowman capsule	SCID mice with glycerol-induced tubulonecrosis: homing of $CD133^+$ cells to kidney tubules	[17]
	Human $CD24^+CD133^+$ cells	SCID mice with acute renal failure: morphological and functional improvement by $CD24^+CD133^+$ cell infusion	[18]
	Human $CD133^+CD24^+PDX^-$ cells	SCID mice with acute renal failure: functional recovery such as reduced proteinuria and glomerular damage	[20]
Renal stem or progenitor cells from renal papilla	Low-cycling proliferating cells (BrdU-retaining cells)	In vitro characterization: heterogeneity of BrdU-retaining cells with epithelial, mesenchymal, and neuronal phenotypes	[22]
	$CD133/1^+nestin^+$ cells	In vitro organ culture: human $CD133/1^+$ cell integration with mouse-derived tubules for new tubule formation	[23]
Renal stem or progenitor cells from renal tubules	BrdU-retaining and $vimentin^+$ cells	I/R rat model: integration of $EGFP^+$ tubular cells with regenerating renal tubules	[27]
	$ALDH^{high}$ cells	$ALDH^{high}$ cells: cell proliferation capability and $CD133^+CD24^+$ expression	[114]
Primary renal cells	Human $CD10^+CD13^+$ cells	Pure population of human renal tubule cells	[33]
	Unpurified human primary renal cells	High percentage of proximal tubular cells (70–80%) with high proliferation capability	[34,35]
	EPO^+ cell population in human primary renal cells	I/R rat model: functional and structural improvement by amelioration of inflammation and oxidative stress	[36]
ESC	In vitro differentiation into renal population [40,41], In vitro organ culture: ES-derived cells integration [42], in vivo mouse: integration of ES cells into proximal tubules [43]		
iPSC	In vitro differentiation into renal population [44,96], in vivo rat AKI: improved survival of animal through enhancement of renal functions [46], in vivo mouse AKI: human iPSCs engrafted into damaged tubules and restored renal function and structure [47]		
AFSC	In vivo renal function improvement in an AKI mouse model [50], an SCID mouse [51], an Alport syndrome mouse model [52], and a kidney autotransplantation model in pig [53]		
BM-MSC	In vivo kidney function improvement in mouse [55,56,58,61] or rat [54,57,59,60] models, clinical trials with kidney transplantation [67,68]		
ADSC	In vivo kidney function improvement in mouse [63], rat [62,64], and pig [65,66] models		

ADSC, adipose-derived stem cell; *AFSC*, amniotic fluid-derived stem cell; *AKI*, acute kidney injury; *ALDH*, aldehyde dehydrogenase; *BM-MSC*, bone marrow-derived mesenchymal stem cell; *BrdU*, 5-bromo-2′-deoxyuridine; *EGFP*, enhanced green fluorescent protein; *EPO*, erythropoietin; *ESC*, embryonic stem cell; *I/R*, ischemic/reperfusion; *iPSC*, induced pluripotent stem cell; *PDX*, podocalyxin; *SCID*, severe combined immunodeficiency.

coordinated balance, this mixed population of cells maintains homeostasis within the renal microenvironment that supports cell-to-cell interactions [13]. Disruption of renal homeostasis occurs after injury or in the case of disease, which in turn elicits the release of cellular and molecular signals to interact with cell components to facilitate regeneration and repair the damaged kidney. Extrinsic regulators within the renal niche initiate renal-specific differentiation and self-renewal of various renal cells and stem cells. Although the existence and identification of renal stem or progenitor cells in adult kidney tissues have been controversial, numerous studies indicate that renal stem and progenitor cells exist in the Bowman capsule, papilla, and tubular sections [7]. Thus, purification and expansion of these cells could be sources for cell therapy to treat renal failure.

The Bowman capsule initiates the filtration of blood and generation of urine [14]. The Bowman capsule is composed of several layers: the parietal layer, the Bowman space, the visceral layer, and a filtration barrier that contains specific kidney cell types. In early studies, a few cell surface markers were identified to isolate renal stem cells from the Bowman capsule. One such marker for cell isolation is the CD133 stem cell marker, which is expressed in hematopoietic stem cells [15] and in the kidney during embryogenesis [16]. Bussolati et al. [17] and Sagrinati et al. [18] isolated and expanded $CD133^+$ cells from normal adult kidney cortex and from kidney tissues of cancer patients obtained at nephrectomy. Although cultured $CD133^+$ cells developed into epithelial and endothelial cell phenotypes [17], $CD24^+CD133^+$ cells displayed various differentiation lineages, including mature proximal and distal tubule formation and adipogenic and osteogenic phenotypes [18]. In preclinical studies, $CD133^+$ [17] or $CD24^+CD133^+$ cells [18] facilitated therapeutic outcomes when administered into renal injury models [17,18].

Glomerular filtration for removal of waste from the blood is one of the kidney's primary functions, and podocytes within the glomerulus have a significant role in the filtration process [19]. However, because podocytes have limited ability for cellular proliferation in the injured kidney, use of stem or progenitor cells that can become more podocytes is an alternative approach for the recovery of normal kidney filtration. Ronconi et al. [20] tested this possibility using $CD24^+CD133^+$ cells. Interestingly, $CD24^+CD133^+$ cells without podocalyxin (PDX) expression ($CD24^+CD133^+PDX^-$ cells) showed tubular and podocyte differentiation, whereas $CD24^+CD133^+PDX^+$ cells contained only podocytes, which suggests that these cells behave as bipotent renal progenitors [21]. Furthermore, in an in vivo study using Adriamycin-induced nephropathy in mice, only $CD24^+CD133^+PDX^-$ cells resulted in functional recovery, including reduced proteinuria and glomerular damage, whereas injection of $CD24^+CD133^+PDX^-$ or $CD24^-CD133^-$ cells was not effective for renal repair.

Another niche for renal stem cells is the renal papilla. This is located in a central region of the kidney that contains epithelia of collecting ducts and the loops of Henle that concentrate urine [7]. In an early study [22], a renal stem-like cell population with a low cell-cycling rate was identified. To track such low-cycling cells in adult kidney, Oliver et al. [22] used a nucleotide bromodeoxyuridine (BrdU) labeling technique to demonstrate that BrdU-retaining cells existed in the papilla. In vitro and in vivo studies demonstrated that BrdU-labeled cells were heterogeneous in nature and included ZO-1–positive epithelial, α-smooth muscle actin–positive mesenchymal, and III-β-tubulin–positive neuronal phenotypes. Based on these results, the authors concluded that the renal papilla contained low-cycling cells and is a niche for adult renal stem cells.

Extending the previous conclusion that CD133-positive cells act as renal stem cells in the Bowman capsule [17], Ward et al. [23] reported that $CD133^+$ cells isolated from adult human papilla and cortex could integrate into tubular structures in their developing stages. Isolated $CD133/1^+$ papilla and cortex cells were positive for nestin, as reported previously by others [24]. A differentiation capability assay revealed that the $CD133/1^+nestin^+$ cells changed to a neurogenic and epithelial-like lineage and underwent tubulogenesis. In a metanephric organ culture, $CD133/1^+$ cells from both the papilla and cortex integrated into mouse-derived tubules during in vitro cell culture, indicating the integration capability of the renal stem cells into the formation of new kidney tubules.

The renal tubule has been considered a renal stem cell niche. It has an important role in reabsorption of electrolytes and proteins and amino acid transportation through hydrolase activity. Previously, the S3 segment of proximal tubule was identified as a renal stem niche in which a significant number of cells were observed to be mitotically active in the region, as confirmed by the expression of proliferating cell nuclear antigen [25]. To identify cellular phenotypes of tubular cells further, several studies used transgenic animals that express reporter markers [26–28]. Lin et al. used transgenic mice expressing enhanced green fluorescent protein (EGFP) protein in mature tubular epithelial cells [27]. After ischemia and reperfusion injury in mice, the investigators found that EGFP-positive tubular cells retaining BrdU labeling and vimentin expression formed regenerating renal tubules, indicating that the intrinsic cell population for renal regeneration is present in the tubule.

To use kidney tissue–derived stem cells for cell-based therapies, efficient isolation and expansion of cell populations from renal tubules are necessary. Lindgren et al. chose a stem cell marker, aldehyde dehydrogenase

(ALDH), which was used to isolate hematopoietic stem cells [29]. ALDH is an enzyme that converts retinol into retinoic acid, and its activities in various tissues [30] are closely related with stem cell proliferation and differentiation. This study reported that tubule cells with high ALDH activity ($ALDH^{high}$ cells) showed $CD133^{+}CD24^{+}$ phenotypes in the human tubule tissue and maintained better cell proliferation capability than $ALDH^{low}$ cells, indicating that $ALDH^{high}$ cells may represent renal progenitor cells from adult human tubules.

Other markers have been used to purify tubular epithelial cells. Using CD10 and CD13, epithelial cell markers [31,32], Van der Hauwaert et al. established a cell sorting method to purify proximal tubular epithelial cells from human renal tissue [33]. Although $CD10^{+}CD13^{+}$ cells comprised only about 4% of the entire cell population, once purified, they showed cell proliferation capability and maintained clear proximal tubular cell phenotypes, such as aquaporin-1 and N-cadherin. On the other hand, $CD10^{-}CD13^{-}$ cells displayed distal and collecting duct markers such as E-cadherin and MUC1; cells positive for CD10 or CD13 were heterogeneous. The investigators concluded that isolation of a pure population of human-derived renal tubule cells is possible, and the isolation method using the markers can be applied to isolate renal stem cells as well.

Our group has focused on isolating and expanding primary kidney cells that could be easily used in clinical applications [34]. After in vitro cell culture, cultivated kidney cells show efficient growth capability. Approximately 70–80% of cultured cells are composed of proximal tubular cells; the remaining cells consist of distal tubular cells, collecting duct cells, podocytes, and other types of cells. Using our established cell culture techniques, we further tested the feasibility of cell therapy using autologous cell sources. We evaluated primary renal cells from diseased kidneys to determine whether their normal phenotypic and functional characteristics are retained, and thus could be used for cell therapy [35]. Renal cells from diseased human kidneys retained characteristics similar to those of normal kidneys, which suggests that autologous cell sources may be used for cell transplantation to treat patients with renal failure [35]. In another study, we purified an EPO-enriched cell population from cultured primary kidney cells and examined the ability of EPO^{+} cells to improve renal function [36]. EPO is a hormone that produces red blood cells and functions as antiinflammatory and antioxidant molecules [37]. In an in vivo study using a rat model with CKD, we demonstrated that EPO^{+} cells ameliorated inflammation and oxidative stress, whereas unpurified cells did not. These results suggest that EPO-enriched cells within primary kidney cells may be a promising cell source for treatment of renal diseases.

Other Cell Sources: Pluripotent, Fetal, or Adult Stem Cells

Various types of cells isolated from nonrenal tissues have been considered potential cell sources, such as pluripotent cells and stem or progenitor cells from fetal and adult tissues. Among pluripotent cells, both embryonic stem (ES) cells [38] and induced pluripotent stem (iPS) cells [39] have been extensively studied in this regard. Both have self-renewable capability and multidifferentiation ability that theoretically is capable of differentiating into any type of cells. ES cells in the kidney have been studied as a potential treatment for renal failure [40–43]. However, ethical dilemmas, danger of cancer from uncontrolled growth, and immunogenic issues still need to be resolved before clinical trials can be initiated.

On the other hand, iPS cells have some advantages in terms of use as an autologous cell source for cell-based therapy compared with ES cells. iPS cells can be obtained by transducing a patient's own fibroblasts with reprogramming genes (Oct3/4, Sox2, c-Myc, and Klf4) [39]. Induction of iPS-derived kidney cells in [44,45] in vitro and some in vivo preclinical studies [46,47] demonstrated the possibility of positive therapeutic effects in treating kidney diseases. However, a sufficiently sophisticated cell culture system for mass cell culture is still needed that can maintain phenotypes and prevent teratoma formation and immunogenicity [48] in vivo.

Fetal and adult tissue-derived stem cells have lower cell growth capability than ES and iPS cells, but the use of tissue-derived cells for therapeutic applications removes the issue of possible teratoma formation. For example, amniotic fluid stem (AFS) cells have been successfully isolated from the amniotic fluid during pregnancy and expanded for therapeutic applications. Our group established a cell culture technique that allowed efficient expansion of AFS cells and demonstrated that the cultured AFS cells showed self-renewal capability and multidifferentiation ability [49]. Preclinical studies using AFS cells demonstrated beneficial outcomes in treating renal failure [50–53].

Stem cells isolated from adult tissues have been extensively studied to treat renal diseases. Mesenchymal stem cells (MSCs), one representative cell source, showed therapeutic effects in renal recovery. MSCs can be easily collected from bone marrow (BM-MSCs) and adipose tissue (ADSCs). Beneficial outcomes for both BM-MSCs and ADSCs in treating renal diseases have been reported in several preclinical models, in which the

application of BM-MSCs [54–61] and ADSC [62–66] varied from acute to chronic renal disease. A few clinical studies using MSC transplantation to improve outcomes of renal transplantation have also been published [67–69].

Engineering of Cell-Based Renal Constructs

The general strategy of engineering a renal construct for treatment of renal diseases is to fabricate functional renal structures in vitro and then implant the constructs for successful integration with host kidney tissues (Table 66.2). To engineer 3D kidney constructs, cultured renal cells are seeded onto a scaffold; the seeded cells within the scaffold are allowed to expand and mature into renal structures. Kidney tissue possesses a complex 3D tubular architecture and is composed of numerous cell types within the renal tissue; therefore, selection of the appropriate cell sources is a consideration for engineering renal constructs. Although many types of renal stem cells have been identified and characterized, use of stem cells as cell sources for engineering renal constructs is still in its infancy for practical applications [7]. Alternatively, most approaches developed for engineering kidney structures have used primary renal cells that are isolated and expanded without further purification. Primary cell culture techniques are now well-established to isolate and expand primary renal cells that demonstrate proliferation and differentiation capability during multiple passages [34].

Another important factor to consider in the in vitro fabrication of kidney structures is the efficiency of engineering the appropriate microenvironment, or niche, to allow seeded cells to form normal kidney-like structures through cell–cell/cell–matrix interactions [70]. Controlled orchestration of engineered niche components, such as the ECM and soluble factors within the scaffolds, is required for the efficient fabrication of renal constructs. Three types of scaffolding systems (natural polymers, synthetic polymers, and biologic tissue matrices) have been used for TE applications [71]. Naturally derived polymers, such as collagen, hyaluronic acid (HA), chitosan, fibrin, alginate, or agarose, provide an alternative to native tissues. Most of these natural biomaterials require biocompatible characteristics with native tissue and can support and guide cellular behaviors such as cell activation, migration, proliferation, and differentiation [72].

Collagen and collagen-based hydrogels have been used extensively to produce scaffolds for kidney TE. Wang et al. [73] developed a renal cell isolation and culture system for engineering renal constructs in vitro (Table 66.2). Glomerular epithelial and mesangial cells isolated from renal tissues were expanded on a collagen-based membrane scaffold (collagen–vitrigel) with a stable, thin shape. The mixture of collagen with vitrigel provided an optimal environment that mimicked the native glomerular structures, which formed a reconstructed glomerular tissue when cocultured with renal cells [73]. In a similar approach, Lu et al. [74] used another collagen-based material that included a Matrigel system containing a mixed population of neonatal rat renal cells to reconstruct 3D renal tissues in vitro. Subsequently, the cells seeded within the 3D collagen hydrogel scaffold self-assembled into engineered renal tissues that contained both tubules and glomeruli-like architecture.

Using the collagen gel scaffold system, our group established a 3D renal-like construct for in vivo study. We found that primary human kidney cells can be expanded in a 3D collagen-based culture system [34], and these cells retained renal phenotypes that stained positively for proximal and distal tubules and collecting duct markers [34]. The cells formed tubule-like structures that provided renal function, such as albumin adsorption. In in vivo implantation studies, cells within the 3D kidney construct maintained their renal phenotypes and remained viable for 6 weeks after subcutaneous implantation in nude mice. These findings demonstrate that human renal cells grown under 3D culture conditions using a collagen gel system are able to generate renal tubule-like structures in vitro and may be used for kidney repair [34].

Another type of ECM contains HA, a glycosaminoglycan, which has been used as a gel-based scaffold for TE applications. HA has a vital role in mammalian development and provides appropriate mechanical properties for scaffold structures [75]. Rosines et al. used a mechanically stable HA construct as a scaffold for in vitro culture and developed a 3D cell culture method to construct renal-like tissues from fetal kidney tissues [76]. HA supported ureteric bud branching, promoted mesenchymal-to-epithelial transformation, and stimulated differentiation of the metanephric mesenchyme and ureteric buds. The authors proposed that HA might serve as an appropriate scaffolding material for kidney TE. Nevertheless, the use of hydrogel-based scaffolds such as HA and other hydrogel polymers is limited by their low mechanical characteristics and high failure rate in terms of shape retention [77].

In an attempt to address these mechanical issues, synthetic biomaterial-based scaffolds have been tested as an alternative source for TE applications. Synthetic polymers include biodegradable polymers such as poly(lactic

TABLE 66.2 Applications to Engineering Renal Constructs and In Situ Kidney Regeneration

Approach Type		Components	In Vitro or In Vivo Animal Model	References
	ECM	**Cells**		
Engineering renal constructs	Collagen/vitrigel	Glomerular epithelial and mesangial cells	In vitro testing to reconstruct renal glomerular tissue	[73]
	Collagen/Matrigel	Neonatal rat renal cells	In vitro testing to reconstruct renal tubules and glomeruli-like structures	[74]
	HA	Fetal kidney tissue	In vitro testing to reconstruct kidney-like structures for kidney development: ureteric bud, metanephric mesenchyme	[76]
	Polycarbonate	Murine kidney cells	Subcutaneous implantation (Athymic mouse), tubules and glomerular structures and functional outcomes (urine-like fluid)	[79]
	PGA	Renal segments	Subcutaneous implantation (Athymic mouse), renal structure formation in the implant	[80]
	PGA	Renal cells from bovine fibroblast using nuclear transfer technique	Subcutaneous implantation (Athymic mouse), tubules and glomerular structures and functional outcomes (urine-like fluid)	[81]
	Decellularized kidney scaffold	Renal cell and EC	Experimental orthotopic implantation using whole-kidney construct (rat)	[84]
	Decellularized kidney scaffold	EC	Experimental orthotopic implantation using whole-kidney construct (pig)	[94]
	ECM	**Bioactive factors or small molecule**		
In situ kidney regeneration	P-collagen	–	Intraperoneal injection into chronic cyclosporine nephropathy of rats, improved renal function through fibrosis reduction	[97]
	PEG-based gel	–	Injection into renal capsule pocket of rats, feasibility test of gel-based drug delivery, no renal damage and normal host cell infiltration	[98]
	Microspheres based on PLLA, PDLA, PEG	Rapamycin	Intracapsule implantation into rat I/R model, compared with subcutaneous injection, reduced fibrotic tissue in renal-injected group	[103,115]
	–	BMP-7	Systemic injection into TGF-β1–induced EMT model in mice, improved renal function	[106]
	–	Antibodies to epididymis protein (HE4)	Systemic injection to progressive renal disease model, reduced fibrosis and improved renal function	[107]
	–	BMP agonist peptide (THR-123) and ACE inhibitor (Captopril)	Intravenous injection and reduction of fibrosis in mouse renal diseases	[111]
	–	HDAC inhibitors (PTBAs analog)	Intraperitoneal injection and reduction of fibrosis in aristolochic acid–induced kidney injury	[94,112]

ACE, angiotensin-converting enzyme; *ADSC*, adipose-derived stem cells; *AFSC*, amniotic fluid-derived stem cell; *BM-MSC*, bone marrow-derived mesenchymal stem cells; *BMP*, bone morphogenetic protein; *CSF-1*, colony-stimulating factor-1; *EC*, endothelial cells; *ECM*, extracellular matrix; *EMT*, epithelial-to-mesenchymal transition; *EPC*, endothelial progenitor cells; *ESC*, embryonic stem cell; *HA*, hyaluronic acid; *HDAC*, histone deacetylase; *HE4*, human epididymis protein 4; *I/R*, ischemia/reperfusion; *iPSC*, induced pluripotent stem cells; *P-collagen*, polymerized collagen; *PDLA*, poly(D-lactic acid); *PEG*, polyethylene glycol; *PGA*, polyglycolic acid; *PLLA*, poly(L-lactic acid); *PTBAs*, phenylthiobutanoic acids; *TGF-β1*, transforming growth factor-β1.

acid), poly(glycolic acid) (PGA), and poly(lactic-*co*-glycolic acid). Owing to a well-controlled degradation profile, nontoxicity, and simple fabrication techniques, the biodegradable polymers have been used as scaffolds sources for renal TE; however, synthetic polymers are limited in terms of slow degradation in the body and may cause inflammatory responses [78].

As an initial approach to testing the feasibility of using synthetic polymers for engineering renal constructs in vitro, our group used PGA scaffolds as an artificial ECM niche to expand rabbit renal cells within the scaffold. Renal cells isolated from proximal and distal tubules and glomeruli were cultured separately and then seeded onto PGA scaffolds. Engineered kidney-like constructs were subcutaneously implanted into athymic nude mice. Nephron-like segments formed within the implant construct, and the seeded cells had proliferation capacity, as shown by BrdU staining. These results demonstrate that cultured primary rabbit renal cells could reconstitute tubular structures with a homogeneous cell type within each tubular tissue.

In another study, our group used a tubular-shaped scaffold prepared from polycarbonate material for 3D culture of murine kidney cells [79]. The tubular-shaped scaffold was connected at one end to a silicone-derived catheter that terminated in a plastic reservoir; this construct was implanted subcutaneously in athymic mice. The implanted construct retained highly organized tubular structures and formed glomerulus-like structures with extensive vascular integration. Furthermore, the tubule-like structures contained renal phenotypes of both proximal and distal tubular cells and cells of the ascending loop of Henle, as confirmed by immunohistochemistry. The newly formed structures exhibited renal function by excreting high levels of solute in a yellow urine-like fluid [79].

Using the same synthetic polymer, a similar study was performed using kidney segments. Kim et al. loaded renal segments onto 3D PGA polymer-derived scaffolds and implanted the renal-like units in vivo [80]. The 3D renal reconstructs that formed contained glomeruli and tubules, which suggested that renal structures can be reconstituted by transplanting renal segments with supporting biodegradable scaffolds.

Lanza et al. bioengineered kidney-like tissues with a nuclear transfer technique by isolating renal cells from early-stage cloned bovine fibroblasts that were then seeded onto synthetic scaffolds derived from PGA [81]. The implanted construct formed highly organized tubular- and glomerular-specific structures that exhibited secretion of urine-like fluid from the implant. Moreover, this study suggested that the ability to generate histocompatible cells using a nuclear transfer technique could address a major challenge in transplantation medicine.

The use of acellular tissue matrices as renal scaffolds is attractive because natural and synthetic polymers cannot replicate the precise spatial architecture of complex structures such as the kidney. In particular, acellular tissue scaffolds are prepared by removing cellular components from the tissues through mechanical or chemical processes to produce collagen-rich matrices [82]. These collagen-rich matrices tend to degrade slowly after implantation and are remodeled with ECM proteins secreted by ingrowing cells. Therefore, much attention has been directed toward developing renal decellularization techniques to produce acellular kidney scaffolds that can maintain native kidney structures such as ECM, bioactive factors, and vascular integrity. Several groups have developed decellularization techniques to produce acellular renal scaffolds to recreate whole-kidney constructs [83,84].

Using biologic scaffolds obtained from the decellularization process, one of the most promising approaches is to bioengineer whole-kidney constructs, in response to the shortage of transplantable kidneys. Progress in whole-organ engineering involving decellularization, followed by recellularization of the resultant collagen-rich scaffolds, has offered a promising approach to creating transplantable organ constructs [85–89]. Decellularization/recellularization techniques have enabled researchers to create bioengineered whole organs in vitro successfully, such as heart [85], lung [89], liver [86,88], and the kidney [84]. Although a few in vivo studies have been performed using small-animal models, these have been limited to short-term assessments; engineered organs function for only a few hours after implantation. An implantation study of a bioengineered rat kidney construct demonstrated the feasibility of the whole-kidney engineering technology based on decellularization/recellularization of kidney tissue [84]. In the recellularization process of acellular kidney matrices, previous studies used ES cells [90,91] and viable kidney explants [92] to recreate engineered kidney structures in vitro [84]. Although these approaches demonstrated the feasibility of using recellularization of a renal matrix to bioengineer a kidney, the field of whole-organ engineering is still in its infancy; several challenges must be addressed before clinical applications are possible.

One such challenge is long-term success in the vascular patency of bioengineered organs in vivo. Without complete and functional endothelial reseeding of vascular matrices, significant thrombosis is likely to occur within the acellular vasculature, which makes the recellularized construct nonfunctional [86,93]. To address this issue, we developed a cell seeding technique that enables efficient endothelial cell coating of the vascular walls of acellular porcine kidney matrices [94]. The acellular renal scaffold was prepared from native porcine kidneys using our previously described decellularization technique [83]. Conjugation of CD31 antibody molecules in the vascular lumen enhanced endothelial cell retention on the vasculature and improved the vascular patency of the implanted scaffold during implantation. These results suggest that endothelial cell seeding plus antibody conjugation could maintain the vascular patency of bioengineered whole kidneys in vivo. However, to make this technology amenable to clinical use, other critical challenges need to be addressed. These include fabricating acellular renal scaffolds on a clinical

scale [83,95], efficient recellularization of scaffolds using clinically relevant cell sources to recreate fully functional kidney constructs [34,44,96], and viable long-term implantation without severe thrombosis [94].

CELL-FREE APPROACH: IN SITU RENAL REGENERATION

An important issue in tissue regeneration in situ is how to fabricate a scaffolding system for efficient recruitment of host cells into the implant and reprogramming of the infiltrated cells within the scaffold. Both natural ECM and synthetic biomaterials have been used as a scaffold to promote in situ renal regeneration [97,98] (Table 66.2).

Whereas collagen-based scaffolds have been widely used as a cell seeding scaffolding system in classical renal TE, several types of collagens have been tested as a natural ECM for renal regeneration in situ. One type of collagen derivative, polymerized collagen (P-collagen), a mixture of pepsinized porcine type I collagen and polyvinylpyrrolidone, facilitated antiinflammatory and antifibrotic properties and reduced scar formation [99,100]. Generally, modulation of inflammation and fibrosis is closely associated with efficient renal regeneration. Sanchez-Pozos et al. used P-collagen to ameliorate renal pathology in a model of chronic cyclosporine A (CsA) nephropathy [97]. P-collagen partially reduced CsA-induced renal pathology and improved anatomic and functional renal properties. These effects were associated with lower levels of inflammatory markers, such as transforming growth factor-β (TGF-β) and Kim-1. In this study, P-collagen functioned as an ECM niche to improve the renal microenvironment by reducing inflammation and fibrosis, thereby improving renal function.

Controlling degradation of the ECM is necessary to maximize therapeutic effects and prevent the rapid disappearance of the added molecules [101]. Serban et al. developed a cross-linked gelatin-based microsphere with continuously tunable degradation; thus, the collagen-derived material served as an ECM scaffold [102]. An in vitro degradation profile showed that the cross-linked gelatin microspheres were resistant to enzymatic degradation. Effects of cross-linking on slow degradation were confirmed by the orthotopic injection of materials into animals. Results of this preliminary study suggested that the product of gelatin-based microbeads might be used to develop materials that catalyze kidney regeneration in the presence of renal disease.

Similarly, synthetic hydrogel systems with different degradation profiles have been developed to assess the feasibility of using them within the kidney for treatment. Dankers et al. synthesized two types of materials with different physiochemical properties based on polyethylene glycol (PEG)-based hydrogels. The hydrogels were injected into the renal capsule pocket to test the feasibility of gel-based drug delivery [98]. The injected synthetic hydrogels did not damage the renal cortex and facilitated normal host cell infiltration of macrophages and myofibroblasts. These strong, flexible, and slow-eroding chain-extended hydrogels might be suitable for the sustained intrarenal delivery of organic drugs. On the other hand, the weaker, softer, and fast-degrading bifunctional hydrogels may be more suitable for the short-term delivery of protein-based drugs to the kidney cortex. These favorable biological behaviors of supramolecular hydrogels make them excellent candidates for subcapsular drug delivery.

For the efficient delivery of such soluble factors and drugs, hydrogel systems have been developed to modulate controlled release of therapeutic drugs [101]. Zandstra et al. [103] developed a drug delivery system based on the use of synthetic monodispersing microspheres to deliver rapamycin, which is known to reduce inflammation in rodent models of kidney disease [104,105]. The microspheres were synthesized using poly(L-lactic acid), poly(D-lactic acid), and PEG, and then rapamycin was codissolved into the copolymer solutions. The rapamycin-incorporated microspheres were administered through subcapsular injection in a rat model of AKI. Therapeutic effects were then compared with subcutaneous injection to mimic systemic administration. Local intrarenal delivery of rapamycin-incorporated monospheres facilitated decreased macrophage infiltration and a significantly lower number of myofibroblasts in the kidney compared with the group with systemic administration. Local delivery of rapamycin caused a transient increase in the deposition of collagen I but not collagen III. The authors concluded that therapeutic effects might increase if rapamycin were delivered in a monosphere subcapsularly; moreover, combination with low systemic concentrations of rapamycin may lead to an effective intrarenal delivery method.

Alternatively, small proteins have been used as soluble factors to improve renal function. For example, bone morphogenic protein-7 (BMP-7), a member of the TGF-β1 family, is highly expressed in the kidney; furthermore, genetic deletion of TGF-β1 in mice results in severe impairment of kidney development. Zeisberg et al. hypothesized that administration of exogenous BMP-7 would enhance repair in severely damaged renal tubular epithelial cells. Results from this study showed that the positive effect by BMP-7 administration was closely associated with the reversal of chronic renal injury through epithelial-to-mesenchymal transition. Collectively, these results provide

evidence of cross-talk between BMP-7 and TGF-β1 in regulating epithelial-to-mesenchymal transition in healthy and diseased kidneys [106].

Similarly, in another study, fibrosis-neutralizing antibodies were administered to alter the fibrotic environment of injured kidneys [107]. Using a transgenic mouse model to track and isolate myofibroblasts, human epididymis protein 4 (HE4) was identified as the most upregulated gene in the fibrosis-associated myofibroblasts. Administration of an anti-HE4 antibody accelerated collagen type I degradation and inhibited fibrosis in three different mice models of renal disease. The authors concluded that HE4 protein is a potential biomarker of renal fibrosis and could be a new therapeutic target [107].

Despite these findings, protein-based therapy is limited by several obstacles, such as long-term pharmacologic resistance. Chemical and pharmacological studies have focused on developing small molecules for tissue regeneration [108,109]. The concept behind this approach is to stimulate the endogenous tissue niche by administering small molecules. Studies of the protein-based method showed that several treatments induced resistance, because antibodies were produced against the administered protein [110]. Thus, using small molecules may prevent this side effect and allow efficient restoration of tissue and organ function.

Several small molecules have been used to treat kidney pathology. For example, Sugimoto et al. [111] focused on the role of BMP in fibrosis formation after renal injury. They showed that THR-123, an agonist of BMP signaling, led to the repair of damaged tubular cells and improved renal function by reducing fibrosis in several renal disease models, such as nephrotoxic serum-induced nephritis, ischemic reperfusion injury, unilateral ureteral obstruction, and diabetic nephropathy [111]. THR-123 plus captopril, an angiotensin-converting enzyme inhibitor, facilitated synergistic therapeutic effects by controlling renal fibrosis. The authors suggested that a synthetic small peptide, such as a BMP-signaling agonist, could be a promising therapeutic agent to treat renal diseases via their control of fibrosis.

Other research in fibrosis reduction relevant to renal regeneration used a small molecule, histone deacetylase inhibitor, which attenuated renal injury through fibrosis reduction [112]. Novitskaya et al. reported that a phenylthiobutanoic acid analog, methyl-4-(phenylthio)butanoate, facilitated renal recovery in a mouse model of aristolochic acid—induced injury [94]. Beneficial effects of the small molecules were confirmed by decreased cell death, proliferation of proximal tubular cells, and decreased macrophage infiltration, which collectively reduced fibrotic tissue and ameliorated renal injury.

CONCLUSIONS AND FUTURE PERSPECTIVES

Developments in TE and RM have shown promising advances in repairing and restoring damage from renal failure. These approaches include cell therapy using autologous and "off-the-shelf" allogeneic cell sources, engineering cell-based renal constructs, and use of an endogenous renal cells and niche for in situ renal regeneration. Such experimental studies have tested new strategies for clinical treatments for kidney disease, and some are clinically applicable [113].

Nonetheless, several challenges need to be addressed to enhance therapeutic outcomes. Continued work is needed to understand the complex milieu of the renal microenvironment (i.e., niche) and to develop renal stem cell culture and expansion techniques to support future cell-based therapies for renal disease. In addition, future studies should address better methods to devise tissue-engineered renal constructs and their safe and effective implantation, determine novel ways to prevent immunologic side effects of cell-based therapies, and address ways to reduce thrombosis and improve innervation of the transplanted cells or engineering of renal constructs.

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CHAPTER

67

Functional Tissue Engineering of Ligament and Tendon Injuries

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INTRODUCTION

Tendons and ligaments are soft connective tissues composed of closely packed, parallel collagen fiber bundles that connect bone to muscle and bone to bone, respectively. These unique tissues serve essential roles in the musculoskeletal system by transferring tensile loads to guide motion and stabilize diarthrodial joints. Injuries to tendons, such as the patellar tendon (PT) of the knee, or ligaments, such as the collateral and cruciate ligaments of the knee, upset the balance between mobility and stability of this joint. These injuries are often manifested in abnormal knee kinematics and damage to other tissues in and around the joint, such as meniscus and articular cartilage, which may lead to morbidity, pain, and osteoarthritis. With the high incidence of ligament and tendon injuries in sports and work-related activities, improvements in healing and repair of these tissues are of great interest [1].

There is dramatic variability in the propensity for healing of the medial collateral ligament (MCL) and anterior cruciate ligament (ACL) in the same knee joint. Clinical and laboratory studies have shown that injuries to the MCL could generally heal well such that nonsurgical management has become the treatment of choice and the structural properties of the femur−MCL−tibia complex (FMTC) in the functional range are restored within weeks [2−8]. However, the mechanical properties of the healed MCL (i.e., the stress−strain relationship) could not return to those of the normal MCL and the histomorphological appearance (e.g., uniform distribution of small collagen fibrils) and biochemical composition are altered (e.g., elevated type III and V collagens) [9−19]. As such, the healing MCL needed to be significantly larger (higher in quantity) to make up for the lack of quality.

For the ACL, it is well-known that a midsubstance tear would not heal and the success of nonsurgical management has been limited. Thus, surgical reconstruction of the ACL using autografts harvested from the PT or hamstring tendons as a replacement is performed to restore knee stability and function. However, there are issues affecting patient outcome from the use of bone−PT−bone (BPTB) autografts, including a persistent palpable defect in the tendon, anterior knee pain, arthrofibrosis, changes to the remaining PT, and PT adhesion to adjacent tissues (i.e., the fat pad) [20,21]. For hamstring tendon autografts, there are issues including a slower healing rate at the soft tissue to bone interphase, less long-term stability of the knee [22], significant hamstring muscle weakness [23,24], as well as an increased prevalence of bone tunnel enlargement after reconstruction [25−28].

Long-term clinical follow-up studies for patients who underwent ACL reconstruction for 10 or more years demonstrate up to 25% of unsatisfactory results that include a prevalence of osteoarthritis [29−31]. Hence, functional tissue engineering (FTE) and regenerative medicine approaches are attractive to improve the suboptimal quality of the healing MCL and issues related to ACL graft harvest and healing after reconstruction. In addition, healing of the injured ACL using FTE as an alternative to reconstruction is a subject of intense interest. These same principles could also be applied to aid the repair of other ligaments and tendons [17,32−37].

In this chapter, we will review the properties of normal and healing ligaments and tendons and discuss the current FTE methods, including the use of growth factors, gene delivery, stem cell therapy, and biological augmentation as well as mechanical augmentation, aimed at enhancing tendon and ligament healing. The goal is not only to restore

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the normal histomorphological appearance, biochemistry, and mechanical properties of the healing ligament, but most important, to regain normal joint function. New technologies and research avenues are suggested that have the potential to enhance treatment strategies for ligament and tendon injuries.

NORMAL LIGAMENTS AND TENDONS

Biology

Ligaments and tendons are hypovascular [38–40] and hypocellular with less than 5% of the total volume occupied by cells [39,41,42]. The cells in these tissues, fibroblasts, or tenocytes, exert important functions in maintaining and remodeling the extracellular matrix (ECM) in which they reside, which consists of both fibrillar (e.g., collagen and elastin) and nonfibrillar (e.g., proteoglycans, elastin, glycolipids, and water) components. The collagen fibers are organized and well-oriented along the longitudinal direction. Normally, the cells are arranged in rows along these fibers [43,44].

Collagen fibers in normal ligaments or tendons are mainly formed by type I collagen (roughly 70–80% dry weight). When viewed histologically under unloaded conditions, these collagen fibers display a crimp pattern with a regular wavy appearance [41,42,45,46]. Under transmission electron microscopy, a bimodal distribution of collagen fibril size can be observed with one group of fibrils measuring 40–75 nm in diameter, and the other, larger fibrils 100–150 nm in diameter [47–50]. Such a bimodal distribution is functionally significant because the incorporation of a high fraction of small-diameter fibrils ensures better interfibrillar binding by virtue of their higher surface–volume ratio whereas the large-diameter fibrils meet the strength requirements. In addition, a bimodal distribution can improve fibril packing—the smaller fibrils wedge themselves in the spaces left among the larger ones [51].

Besides type I collagen, many other collagen subtypes, including III, V, X, XI, and XII, are present in much smaller amounts but still play important roles in maintaining the structure and functions of ligaments and tendons. For example, type III collagen is involved in tissue healing and remodeling [52]; type V collagen has been found to exist in association with type I collagen and serve as a regulator of collagen fibril diameter [53,54]; and type XII collagen provides lubrication between collagen fibers [55]. Interestingly, collagen types IX, X, and XI have been identified to coexist with type II collagen at the fibrocartilaginous zone of the ligament–bone and tendon–bone interfaces [56–58]. The significance of the coexistence has been related to the minimization of stress concentrations when loads are transmitted from soft tissue to bone [59,60].

The ground substance makes up only a small percentage of the total dry tissue weight of ligaments or tendons but it is nevertheless pivotal in its function because of its ability to intake water. Water and proteoglycans are crucial to the gliding function of fibers in the tissue matrix by providing lubrication and spacing of collagen fibers. Elastin, another fibrillar macromolecule in matrix, is also present in ligaments and tendons at a few percent by weight. Although its detailed significance has yet to be elucidated, elastin has been implicated in its ability to allow the tissue to return to its prestretched length and shape upon unloading. Collectively, these constituents are indispensable in the normal functioning of collagen fibers by distributing load optimally in response to mechanical stress.

Although ligaments and tendons are morphologically similar, they are different in their compositions. For example, ligaments are composed primarily of water (65–70% of wet weight) and collagen (70–80% of dry weight); type I collagen is the most abundant collagen subtype. Type III collagen (8% dry weight), type V collagen (12% dry weight), and other minor subtypes such as II, IX, X, XI, and XII, have also been found to be present [56–58,61]. Tendons, on the other hand, generally contain less water (55% wet weight) and slightly more type I collagen (85% dry weight), along with much smaller amounts of other collagen subtypes, such as III, V, XII, and XIV [49]. In addition, most ligaments are more metabolically active. They have more cellular nuclei, a higher DNA content, and greater amounts of reducible cross-links between collagen fibers [45].

Biomechanics

The major function of ligaments and tendons includes maintaining the proper anatomic alignment of the skeleton and guiding joint movements. They also transmit forces along their longitudinal axis; hence, their biomechanical properties are measured in uniaxial tension and they exhibit nonlinear behavior governed by the recruitment of collagen. These unique properties allow ligaments to maintain smooth normal joint motion that requires low loads. In response to high loads, stiffness increases dramatically to limit excessive joint displacements. Thus, when large

muscle forces are applied, the bones are well-aligned to allow them to glide smoothly on each other. Ligaments and tendons also exhibit time- and history-dependent viscoelastic behavior that could be attributed to the complex interactions of tissue constituents such as collagen, proteoglycans, water, and ground substance [62–65]. Viscoelastic properties of ligaments and tendons are important and clinically relevant. For instance, during regular activities such as walking and jogging, tissues have the ability to soften over time. This phenomenon reduces susceptibility to damage related to fatigue [63–65].

However, after injury, ligaments and tendons generally fail to recover their normal mechanical and viscoelastic properties. Thus, abnormal joint kinematics may result that can lead directly to excessive forces on surrounding tissues (e.g., articular cartilage and meniscus in the knee). This excess loading could result in further injury causing degeneration such as osteoarthritis. Because the ultimate goal is to restore the properties of ligaments and tendons, and thus the function of the injured joint, it is necessary to understand their normal mechanical behavior and how they contribute to joint function. To do this, two common mechanical tests have been designed: (1) uniaxial tensile testing, which is a test to measure the structural properties of the bone–ligament–bone complex and mechanical properties of the tissue substance; and (2) functional testing, which determines the contribution of the ligament or tendon to joint kinematics and the in situ forces in response to externally applied loading conditions.

Uniaxial Tensile Testing

Tendons are generally long and can be tested in their isolated state using sinusoidal-shape or frozen grips to limit slippage. Isolated ligaments, on the other hand, are shorter in length, which makes it difficult to clamp them independently. Hence, a uniaxial tensile test is generally conducted on the entire bone–ligament–bone complex (e.g., FMTC) with tissue insertion sites left anatomically intact. With cross-sectional area (CSA) measurements and the use of tissue markers to measure tissue strain, the structural properties of the bone–ligament–bone complex as well as mechanical properties of the ligament substance can be measured from a load to failure test [66,67].

Structural properties (Fig. 67.1) of the bone–ligament–bone complex (i.e., a load–elongation curve) are generally described by four parameters including stiffness (slope of the linear portion of the load–elongation curve), ultimate load (maximum load at which the complex fails), ultimate elongation (elongation corresponding to the maximum load), and energy absorbed at failure (area under the curve to the maximum load). These data reflect the behavior of the entire bone–ligament–bone complex, which includes tissue size, orientation of collagen fibers to applied loads, and as the contribution of the bony insertions [67].

Measuring the mechanical properties (Fig. 67.2) of the ligament substance (i.e., a stress–strain curve), on the other hand, requires knowledge of the CSA of the ligament, commonly measured using a laser micrometer system [66], and tissue strain, commonly measured using video techniques to track two or more reflective markers placed on the tissue midsubstance [8]. Stress in the tissue is obtained by dividing load by the CSA, and strain is obtained by computing change in the marker distance during the test relative to their original distance. Parameters describing the mechanical properties of the ligaments and tendons (Fig. 67.2) include the tangent modulus (slope of the linear portion of the stress–strain curve), tensile strength (stress at failure), ultimate strain (strain corresponding to the tensile strength), and strain energy density (area under the stress–strain curve until failure). These data represent the quality of the tissue, irrespective of tissue size.

The viscoelastic properties of ligaments and tendons include stress relaxation (decrease in stress over time in response to a constant elongation) and creep (increase in elongation over time in response to a constant load). In addition, they display a phenomenon called "hysteresis" in response to cyclic loading (Fig. 67.3). This results from a loss of

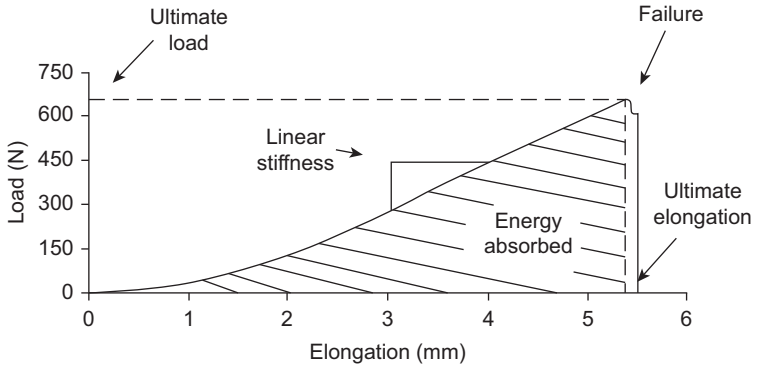

FIGURE 67.1 Typical load–elongation curve representing the structural properties of the femur–anterior medial bundle–tibia complex of the human anterior cruciate ligament.

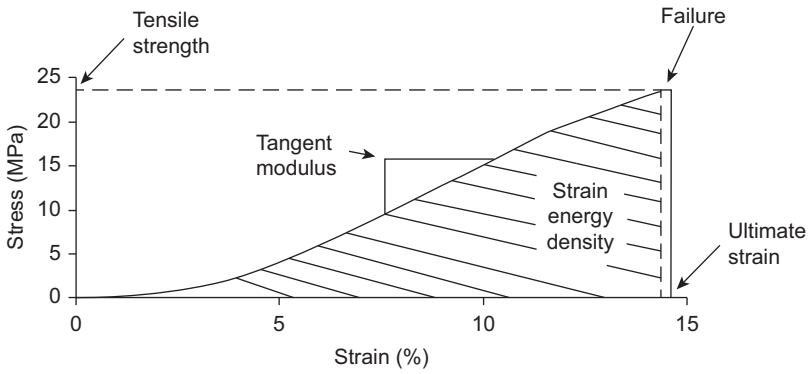

FIGURE 67.2 Atypical stress–strain curve representing the mechanical properties of the anterior medial bundle of the human anterior cruciate ligament.

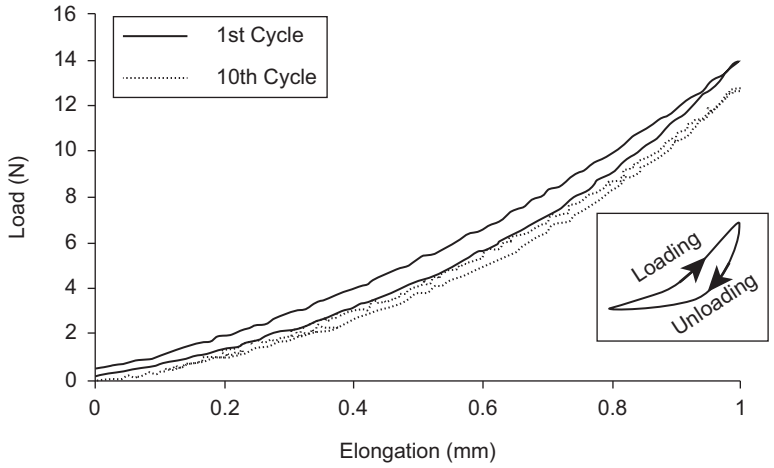

FIGURE 67.3 Hysteresis loops (first and 10th cycles) obtained from cyclic loading of the femur–anterior medial bundle–tibia complex of the human anterior cruciate ligament in uniaxial tension. The area of hysteresis (area between the loading and unloading curves) decreases with repetitive cycling, demonstrating the phenomenon of "preconditioning."

internal energy causing the loading and unloading paths to be different. The area of hysteresis reduces as the tissue undergoes several cycles of loading and unloading and the tissue is said to be "preconditioned," a state desired for a tissue before mechanical testing. Nonlinear viscoelastic models such as the quasilinear viscoelastic theory:

$$\sigma(t) = \int_{-\infty}^{t} G(t-\tau)\frac{\partial \sigma^e(\varepsilon)}{\partial \varepsilon}\frac{\partial \varepsilon}{\partial \tau}\partial\tau, \tag{67.1}$$

and single integral finite strain theory:

$$\mathrm{T} = -\mathrm{p}\boldsymbol{I} + \mathrm{C}_0\{[1+\mu \boldsymbol{I}(t)]\boldsymbol{B}(t) - \mu \mathbf{B}^2(t)\} - \mathrm{C}_0(1-\gamma) * \int_0^t \dot{\mathrm{G}}(t-s)\{[1+\mu \mathbf{I}(s)]\mathbf{B}(t) - \mu \mathbf{F}(t)\mathbf{C}(s)\mathbf{F}^T(s)\}ds, \tag{67.2}$$

have been used to model these behaviors in ligaments and tendons [62–65,68].

These basic testing methodologies described have been employed for a number of decades. In our research center, much work has been devoted to finding the most appropriate testing procedures, which include specimen orientation [69], handling, storage, and hydration [70,71]. These carefully developed methodologies have led to important findings regarding physiological changes associated with growth and development [69–71], the adaptation of ligaments and tendons to mobility [5,6,63–65,72], and the effects of injury and treatment [5,6,19].

Contribution to Joint Function

Joint motion is governed by the direction and magnitude of externally applied loads, ligament forces, contact between joint surfaces, and muscle activity. For the knee, motions include a combination of translations (proximodistal,

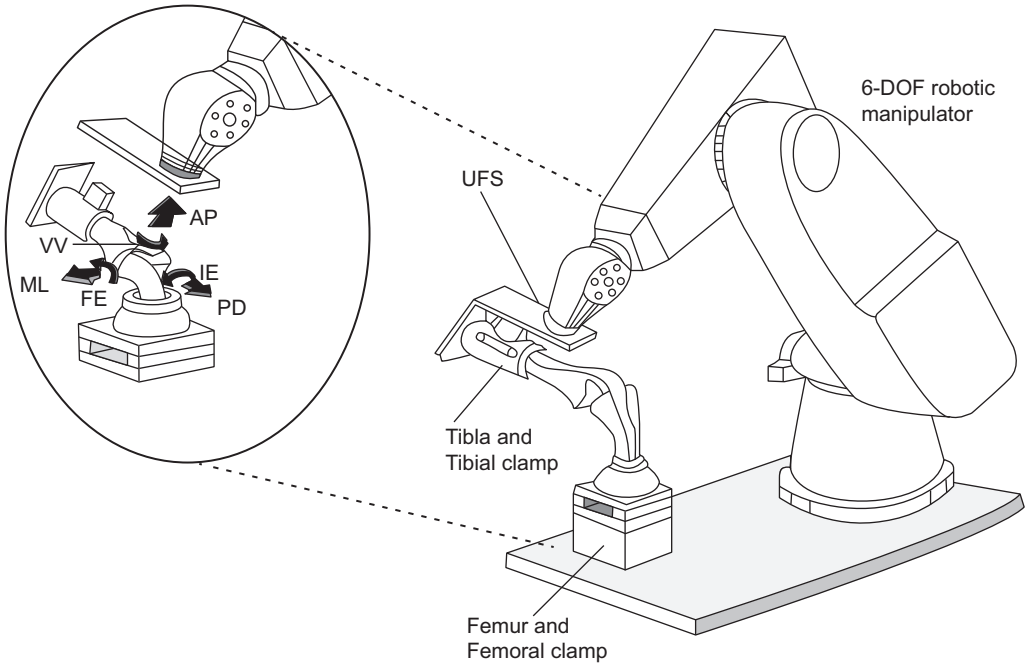

FIGURE 67.4 Schematic drawing illustrating the six degrees of freedom of motion of the human knee joint, indicating anteroposterior (AP), mediolateral (ML), and proximodistal (PD) translation as well as flexion−extension (FE), internal−external (IE), and varus−valgus (VV) rotation. *DOF*, degrees of freedom.

mediolateral, and anteroposterior) and rotations (internal−external, flexion−extension, and varus−valgus). In total, these translations and rotations describe motion in 6 degrees of freedom (DOF).

While evaluating joint function, constraining DOF of the knee can have significant impact on the results obtained [73,74]. When knee motion was allowed in all directions, sectioning the MCL resulted in only small increases in valgus laxity (21%), which suggests that the ACL has a significant role as a joint restraint to this knee motion. However, when anteroposterior translation and internal−external rotation were constrained, valgus laxity increased significantly (171%) after sectioning of the MCL. For this reason, it is important to have a testing device that allows for unconstrained knee motion.

For more than 2 decades, a robotic−universal force-moment sensor (UFS) testing system developed by our research center has been used to study knee kinematics as well as directly measure the in situ forces in the knee ligaments in response to external loading conditions (Fig. 67.4) [75,76]. This methodology has been employed to study the function of the ACL and a number of variables for ACL reconstruction, including surgical technique [77−79] and graft choice [80]. In addition, knee function after ligament injury has been studied for both the MCL and ACL [8,81−84].

HEALING OF LIGAMENTS AND TENDONS

The events of healing of ligaments and tendons can be roughly divided into four overlapping phases: hemorrhage, inflammation, repair (proliferation), and remodeling. Minutes after the ligament injury, blood collects and forms a platelet-rich fibrin clot at the injury site and the hemorrhagic and inflammatory phases occur over several days. In the hemorrhage phase, a cascade of cellular events occurs that includes release of cytokines within the clot followed by the appearance of polymononuclear leukocytes and lymphocytes. These cells respond to autocrine and paracrine signals to expand the inflammatory response and recruit other types of cells to the wound [85].

The reparative phase follows over the next couple of weeks to months. During this phase, fibroblasts recruited to the injury site start forming healing tissue. Growth factors, including transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) isoforms, are involved in modulating healing [86]. Meanwhile, increased neovascularization brings in circulating cells and nutrients to enhance the healing process further. The blood clot quickly turns into newly formed healing tissue that is composed of an aggregation of cells surrounded by a matrix.

However, its histomorphological appearance and biochemical composition are different from those of an uninjured ligament. Notably, there is a homogeneous distribution of smaller-diameter collagen fibrils, which is in stark contrast to the bimodal distribution of the normal ligament [12,15,50]. Biochemically, it contains elevated amounts of proteoglycans, a higher ratio of type V to type I collagen, and a decrease in the number of mature collagen cross-links.

Then the remodeling phase follows for months to years after the injury. In this phase, cellularity and levels of collagen type III are decreased, and the matrix is realigned in response to loading applied to the tissues. On the other hand, the diameter of collagen fibrils remains small, and the level of collagen type V remains elevated for years after injury [9,11,12,15,16]. Type V collagen has been shown to have a central role in regulating the lateral aggregation of smaller collagen fibrils. Thus, elevated type V collagen could be associated with the inferior mechanical properties of healing tissue [87,88].

Medial Collateral Ligament of the Knee

The healing process of the MCL follows this general healing pathway, as described earlier. Thus, it serves as a good model for studying histological, biochemical, and biomechanical changes over time. It has been shown that the process of MCL healing is greatly affected by treatment [5,6,89–92]. Laboratory and clinical studies have shown that controlled mobilization is superior to immobilization [5,6,19,93]. As a result, nonoperative repairs have a better outcome than surgical repairs. In our research center, a severe "mop-end" injury model of the rabbit MCL that tears the midsubstance while damaging the insertion sites was developed and used to compare nonoperative treatment without mobilization with surgical repair with a brief period of mobilization [19]. After 12 weeks of healing, there were no significant differences in varus–valgus knee rotation, in situ force of the MCL, or tensile properties between repaired and nonrepaired MCL [19]. Based on these studies, clinical management shifted from surgical repair with immobilization to nonoperative management (i.e., bracing) with early controlled range of motion exercises as soon as pain subsides [94,95].

The MCL heals with nonoperative treatment, and the stiffness of the healing FMTC begins to approach normal levels, but the CSA of the healed tissue continues to increase with time, measuring as much as 2.5 times its normal size by 52 weeks after injury [93]. Meanwhile, the mechanical properties of the healing MCL remain consistently low compared with those for the normal ligament and do not improve with time. In other words, the healing process involves making a larger quantity of lesser-quality ligamentous tissue. Moreover, studies show that the rate of healing of the ligament is asynchronous with the insertion sites because of its anatomical and morphological complexity.

There is also evidence that the activity level could influence the rate of healing [81–83]. A goat model was used because it has more robust activity, and it is larger than the rabbit. With this model, the stiffness and ultimate load of the healing goat FMTC are closer to control values at earlier periods than those from the rabbit model.

Anterior Cruciate Ligament of the Knee

A midsubstance tear of the ACL has limited potential to heal on its own and can lead to chronic knee instability and damage to secondary stabilizers such as the meniscus. As a result, surgical reconstruction has become the treatment of choice. With the ultimate goal of ACL reconstruction being to restore knee function, the success of these procedures depends on a number of surgical, biomechanical, and biological factors. The most popular choice is autografts from the PT, i.e., bone central (or medial) third of the BPTB, or semitendinosus plus gracilis tendons. Allografts, including the Achilles tendon, BPTB, and hamstring tendons, have seen limited use except in revision surgery or for multiple ligamentous injuries. BPTB grafts are generally considered the reference standard for ACL reconstruction because they facilitate better initial fixation for bone-to-bone healing inside bone tunnels [96–101]. However, the major drawback is that the defect after the large incision does not completely heal for months [20,102–104]. This contributes to a higher incidence of complications including donor site morbidity, patella baja, arthrofibrosis, adhesion to the fat pad, and patellofemoral pain [105–110].

Studies designed to examine the healing PT after harvest have found a deterioration of structural properties of the remaining BPTB complex with a concomitant increase in the CSA of the PT tissue [111–115]. In the rabbit model, the ultimate load of the entire BPTB complex decreased by 38% [112] whereas there was an increase in CSA of 83–108% at 12 weeks after harvest [111,115]. For the central healing tissue, its tangent modulus and ultimate tensile strength were only 15% and 18% of controls, respectively, after 26 weeks [111]. The mechanical properties of the remaining PT tissues also deteriorated compared with sham controls after 24 weeks [115].

After implantation, the autograft becomes inflamed and necrotic, leading to a decrease in graft stiffness and strength [116]. The graft undergoes revascularization and repopulation with fibroblasts followed by remodeling with restructure of the collagen fibers and proteoglycans [117]. Bone block healing within the femoral and tibial tunnels is complete by 6 weeks, whereas graft healing is incomplete, causing consistent failure to occur as the tendon is being pulled out from the tibial tunnel [118]. Over time, however, the femur−graft−tibia complex shows improvement with complete incorporation even though its structural properties fail to be restored to levels of the intact femur−ACL−tibia complex (FATC) even after 12 months [119−121]. Thus, accelerating graft incorporation and healing that lead to an earlier return to normal and sports activities has become a goal of FTE efforts. We will discuss the state of events later in this chapter.

In addition to the graft selection, a number of important surgical decisions include tunnel placement, graft tension, and fixation. A substantial amount of research has focused on these variables in both animal and human models [78,81−83,122]. All of these parameters can lead to various degrees of graft tunnel motion that may affect graft integration and graft healing. Ultimately, these factors have an impact on postoperative rehabilitation and recovery as well as when to return to normal activities and sports.

Multiple Ligamentous Injuries in the Knee

Combined ACL and MCL injuries frequently occur. The ideal treatment is still controversial. Some surgeons elect to reconstruct the ACL surgically without addressing the MCL whereas others advocate reconstruction of the ACL with repair of the MCL. Regardless of the treatment modality, clinical and basic science studies continue to show that the outcome of this injury is worse than for an isolated MCL injury but not as clear compared with an isolated ACL injury.

Our research center has elucidated the effects of ACL deficiency on the healing of the injured MCL using canine, rabbit, and goat models [81−83,92,123,124] and recommended that only ACL reconstruction be performed. Furthermore, repairing the MCL in combination with ACL reconstruction resulted in reduced valgus laxity and improved the structural properties of the FMTC but only in the very early stages. In the longer term, all positive effects diminished [123,124]. To date, surgeons prefer to perform ACL reconstruction after a combined ACL−MCL injury.

Using a larger animal model, i.e., the goat stifle joint, the function of the knee and quality of the healing MCL after a combined ACL−MCL injury treated with ACL reconstruction was examined [81−83]. It was found that the valgus rotation of the stifle joint was twice that for an isolated MCL injury. Moreover, the structural properties of the FMTC and tangent modulus of the MCL substance were substantially lower than those for the isolated MCL injury [8,81−83]. These results further demonstrate a clear need for new treatment strategies to enhance ligament healing after multiple ligament injuries.

APPLICATION OF FUNCTIONAL TISSUE ENGINEERING

FTE emphasizes the importance of biomechanical considerations in the design and development of cell and matrix-based implants for soft and hard tissue healing. Ligaments and tendons are accustomed to being mechanically challenged; therefore, tissue engineered constructs used to replace these tissues after injury or disease must meet these demands. By combining the fields of molecular biology, biochemistry, and biomechanics, novel therapeutic approaches (e.g., growth factors, gene transfer/gene therapy, cell therapy, and biological scaffolds) offer new potential for better treatment. The following is a brief review of available approaches to enhance ligament and tendon healing.

Growth Factors

The application of exogenous growth factors is based on the premise that they can promote healing that will lead to a biologically and biomechanically superior healed ligament substance. Many in vitro and in vivo studies have tried to examine their roles and determine appropriate strategies for their use.

In Vitro Studies

Cell culture or tissue explant methodologies have been the major study designs. By adding exogenous growth factors, responses including cell proliferation, synthesis of ECM proteins such as collagen, proteoglycans, tissue

remodeling enzymes, and cell migration or chemotaxis were measured and compared [125–131]. Early studies in our research center measured the effects of eight different growth factors on the MCL and ACL fibroblast culture [123,132–134]. For cell proliferation, PDGF-BB, epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF) were found to have a significant effect on cell proliferation and caused greater proliferation in MCL fibroblasts versus ACL fibroblasts [134]. The proliferation of MCL and ACL fibroblasts from skeletally immature rabbits actually increased by 7.6 times in response to EGF and 5.6 times in response to bFGF [123]. In skeletally mature rabbits, the insulin-like growth factor and bFGF had significant effects on fibroblast proliferation in both cell types, but the difference was much less pronounced [134]. Other authors had shown the effect of TGF-β1 on ACL fibroblast proliferation to be dose-dependent and smaller doses could act synergistically with PDGF [135]. However, higher concentrations of TGF-β1 inhibited the stimulatory effect of PDGF. These findings suggest complex interactions of growth factors and their potential role in enhancing the proliferation of ligament fibroblasts.

In terms of protein synthesis, TGF-β1 was most effective in collagen synthesis. In MCL and ACL fibroblasts, the level of increase was 160% over controls; most of this increase was for type I collagen [133]. These data suggest that TGF-β1 may improve ligament healing by increasing matrix synthesis during the proliferative and remodeling phases and confirm what was found by other investigators [135].

In vitro models, however, are limited in the extent that they cannot reproduce the complex interplay of signals affected by growth factors in the intricate process of ligament or tendon healing where there is a highly integrated biochemical network of cell signaling events with intrinsic stimulatory and inhibitory feedback loops. Thus, in vivo studies are needed to examine the interaction of biology and biomechanics and the degree to which the healed ligament or tendon substance could restore the biomechanical properties to those for the native tissue.

In Vivo Studies

Based on in vitro studies, EGF and PDGF-BB have the greatest effect on ligament fibroblast proliferation whereas TGF-β1 better promotes ECM production. These growth factors were then applied at different dosages, in isolation and in combination, for an MCL injury in the in vivo rabbit model. It was found that only a higher dose of PDGF-BB improved the structural properties of the FMTC [75,76]. A lower dose of PDGF-BB had little effect, which demonstrated that the effects of PDGF-BB were dose-dependent. However, the mechanical properties of the ligament substance remained unchanged from untreated controls, which demonstrated that the improved structural properties resulted from the formation of a larger quantity of tissue (instead of improved tissue quality). Other investigators showed that higher doses of PDGF could improve the structural properties of the healed ligament [136]. However, administration of PDGF for more than 24 h after the injury markedly decreased its efficacy.

One possible approach to improving the in vivo application of growth factors and cytokines could be to combine it with gene transfer technology to extend their effectiveness with time. Adenoviral bone morphogenetic protein-2 (AdBMP-2) delivered to the bone–tendon interface using a gene transfer technique has been shown to improve the integration of semitendinosus tendon grafts in rabbits [137]. The stiffness (29.0 ± 7.1 versus 16.7 ± 8.3 N/mm) and ultimate load (108.8 ± 50.8 versus 45.0 ± 18.0) were also significantly increased in specimens with AdBMP-2 compared with untreated controls.

Based on these studies, an optimal therapy of introducing growth factors to injury sites is still an open question. The timing of application, mode of delivery, dosage, inclusion of scaffolds and/or cells, and interactions among these variables remain major hurdles. As an example, the delivery of stem cells transduced to express BMP-2 had adverse effects on rotator cuff repair [138]. As a result, investigations have been devoted to therapies such as platelet-rich plasma (PRP) and ECM bioscaffolds, which contain a number of bioactive factors [84,139]. In addition, it is well-recognized that stem cell delivery could modulate the healing response via trophic factors produced by the cells and not by engraftment of those cells [140]. Thus, these therapies could offer highly translational approaches to growth factor delivery.

Gene Therapy

Gene therapy offers an exciting approach to improving ligament and tendon healing. Foreign nucleic acid gene transfer can be introduced into cells to alter protein synthesis or induce the expression of therapeutic proteins. Modern gene therapy relies on mammalian viruses and cationic liposomes as delivery vectors, and both have been developed to deliver genes into host tissue via direct (in vivo injection) and indirect methods (in vitro transduction).

In our research center, we sought to determine whether genes could be transduced into MCL and ACL fibroblasts and whether ligament injury affected gene transfer and expression [141]. Both direct and indirect methods using adenovirus and BAG retrovirus, respectively, were employed. It was found that both techniques resulted in expression of the LacZ marker gene by fibroblasts from intact as well as injured ligaments. Gene expression lasted longer (6 weeks) with the direct method compared with the indirect technique (3 weeks). Fibroblasts from injured ligaments showed transduction in both the wound site and the ligament substance. There was no difference in the duration of gene expression by fibroblasts from intact and injured ligaments, which suggested that injury does not affect gene transfer or expression [141].

Antisense gene therapy that could block the transcription or translation of specific genes that are excessively expressed within healing tissue has also been studied. By binding antisense oligodeoxynucleotides (ODN) to target DNA, the efficacy of using ODNs to regulate the overproduction of collagens III and V was studied [35,142]. Normal human patellar tendon fibroblasts were transfected with antisense collagen III or V ODNs by mixing with lipofectamine. The uptake of ODNs was detected as early as 1 h and as late as 3 days after delivery. The relative expression of collagen V messenger RNA (mRNA) was reduced to $67.8\% \pm 5.1\%$ of missense levels. Also, reverse transcriptase–polymerase chain reaction results showed that the inhibitory effects of the collagen III antisense ODNs were most dominant at day 1, because the type III collagen mRNA level was $38.9\% \pm 19.6\%$ of missense controls. At days 3 and 7, differences could no longer be observed. These results suggested that antisense gene therapy can be a potential FTE approach to enhance the quality of healing ligaments and tendons.

Despite these promising results, several obstacles impede the practical implementation of gene transfer as a biological intervention for ligament healing. The immune reaction against these antigens decreases the expression of the introduced gene [143]. In addition, retroviral infection of fibroblasts often leads to shut-off of the promoter region, which adversely affects expression of the incorporated gene [144]. Thus, delivering ODNs to the appropriate target and reproducibility of the results remain great but exciting challenges.

The literature has shown other techniques such as the use of lentivirus for gene transfer. For example, bFGF was transfected into bone marrow–derived cells (BMDCs) and then implanted into the Achilles tendon [145]. However, these investigators did not observe an enhanced healing response from the injection of stem cells. Also, lentivirus–tumor necrosis factor-α (TNF-α)–RNA interference–expressing TNF-α small interfering RNA was injected in the rotator cuff tendon in a rat model [146]. Thus, the inflammatory response was attenuated by the gene therapy after a chemical insult.

Novel strategies including the search for more effective and less immunogenic vectors, modification of promoters to ensure gene expression after incorporation, and temporary and self-limiting gene expression regulation tailored to the changing environment continue to evolve in gene transfer to aid in ligament healing. Technologies such as CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/CRISPR-associated protein 9 could become a new strategy. As the complex steps involved in gene expression and regulation are further elucidated, the potential therapeutic efficacy of gene transfer is likely to find clinical application.

Cell Therapy

Cell therapy is another potential approach to enhancing ligament and tendon healing because the use of autogenous cells would minimize the immune response. Studies have focused mainly on the application of mesenchymal stem cells (MSCs), specifically BMDCs and synovial tissue-derived fibroblasts into the healing site [147,148]. BMDCs have been shown to have an important role in wound healing [149–151] and can be obtained in higher numbers with relative ease [152]. BMDCs combined with PRP were used to treat rabbit ACL after reconstruction, which resulted in improved structural properties after 8 weeks of healing [153]. The treated tissues with combined PRP and BMDCs had a more mature bone–ligament interface than those in the untreated controls and PRP-only group. Another study attempted transplantation of allogeneic tendon-derived stem cells to rat PT and observed improvement in healing at 16 weeks after injury [154].

Alternatively, fibroblasts, myoblasts, and bone marrow cells have been transplanted into injured ligaments after the induction of marker genes or stimulation by growth factors in vitro. However, issues remain regarding cell therapy. The number of MSCs from bone marrow are relatively low and decrease after transplantation. Thus, it is essential to develop novel in vitro techniques to expand MSCs without altering their differentiation potential. Furthermore, other methods, i.e., bioscaffolds, may be designed to prevent the loss of these cells and to sustain the positive effects.

HEALING OF LIGAMENTS AND TENDONS

The Use of Scaffolds

An empirical method to improve the healing of ligaments and tendons is to use scaffolds to augment the injured part or bridge defects. An ideal scaffold should provide a suitable biological environment for cells to migrate into it and then proliferate, an appropriate mechanical cue to guide the formation of the newly synthesized ECM, and a built-in property to degrade slowly such that the newly formed matrix could take over in bearing the mechanical loads. Both synthetic and naturally occurring biological scaffolds have been used in FTE of ligament and tendon healing [155–158]. Major advantages of using synthetic polymers as scaffolds are their ease of fabrication and reproducibility. A structure can be created that mimics the structure of a ligament or tendon, and appropriate bioactive factors (i.e., growth factors) can be incorporated into the scaffold during manufacturing. However, because the performance of synthetic grafts has been found to be less than satisfactory [159,160], the focus has been placed on the supplementary use of cells, including BMDCs and fibroblasts [161,162].

Alternatively, a number of naturally occurring biological scaffolds, e.g., bovine pericardium (Integra Life Science), human dermal collagen (Alloderm), and porcine small intestinal submucosa (SIS), have been used after proper decellularization and sterilization. Among these scaffolds, SIS products have been approved by US Food and Drug Administration and are widely used in the field. Because SIS contains a large amount of collagen (over 90%) and has a preferred collagen alignment [163], it has the potential of acting as a contact guide to promote cells to deposit new matrix in a more aligned manner [164–167]. As a result, the mechanical and viscoelastic properties of the neotissue are improved. When used, preferably as a noncross-linked biodegradable scaffold, SIS degrades gradually when implanted in vivo. The pace of the degradation not only allows gradual replacement of the scaffold with the newly synthesized host matrix, it controls the timely release of degradation products that are biologically active and beneficial to healing [167a,168,170]. Moreover, SIS entraps a certain amount of bioactive agents (growth factors, fibronectin, and so on) that can be released during degradation to enhance healing [171–173].

Medial Collateral Ligament and Patellar Tendon Healing With Extracellular Matrix (Small Intestinal Submucosa)

Multidisciplinary studies were performed to determine the effect of SIS treatment on the healing of an injured MCL (with a 6-mm gap) using a rabbit model [61,174]. Compared with the untreated group, SIS treatment resulted in improved histomorphological appearance, mechanical properties, and biochemical compositions of the healing MC; they were closer to those of normal ligament in both the short and long term (12 and 26 weeks) (Fig. 67.5). Specifically, SIS treatment further restored the heterogeneous distribution of collagen fibrils, because both large and small fibrils could be found compared with the persistently homogeneous small collagen fibrils in the untreated groups (Fig. 67.6). Accordingly, the CSA of the healing ligament in the SIS-treated group concomitantly decreased by 28%, indicating improved tissue quality. Indeed, improved mechanical properties of the healing MCL were

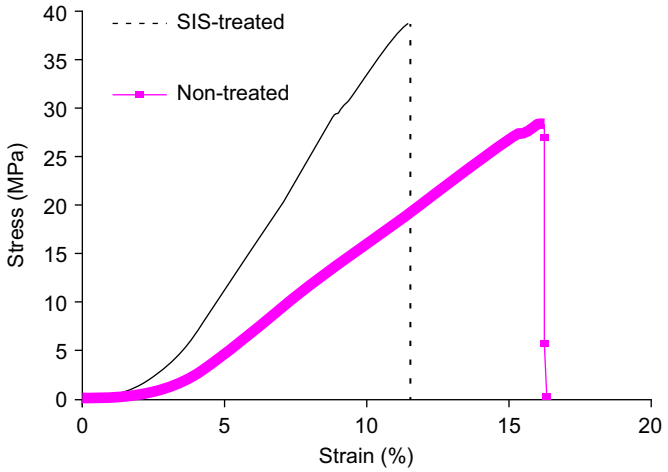

FIGURE 67.5 Typical stress–strain curves for small intestinal submucosa (SIS)-treated and untreated groups at 12 weeks after injury.

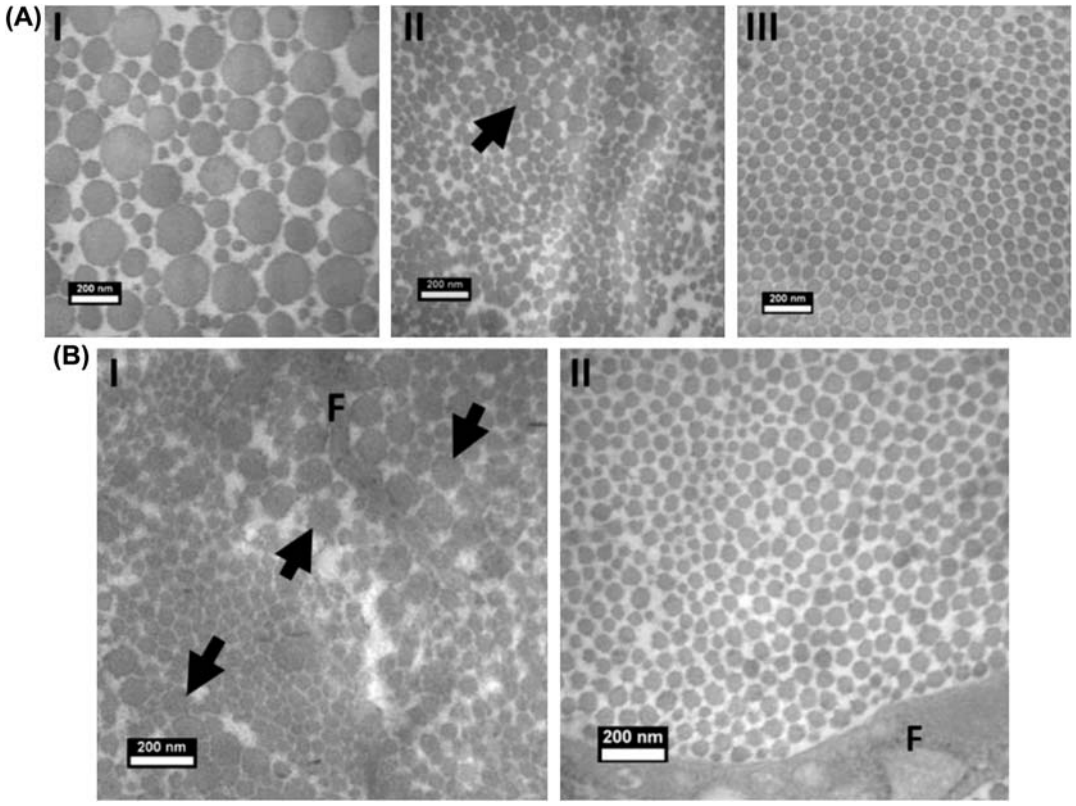

FIGURE 67.6 Transmission electron micrographs (×70,000) of collagen fibrils in (A) sham-operated medial collateral ligament (MCL) (I), small intestinal submucosa (SIS)-treated MCL (II), and untreated MCL (III) at 26 weeks after injury. *Arrow* indicates the appearance of large fibrils between cells in the SIS-treated MCL. (B) Transmission electron microscopy appearance of both large and small fibrils (heterogeneity) in the pericellular area in the SIS-treated MCL (I) and untreated MCL (II). *Arrows* indicates the large fibrils surrounding a cell process. *F*, fibroblast.

found, because the tangent modulus increased by 33% and stress at failure increased by 49% [174]. These positive findings in morphology and biomechanics were mechanistically associated with better collagen organization as well as the regulation of collagen subtypes by a number of small leucine-rich proteoglycans, decorin, lumican, and biglycan, and so on, to reduce the collagen type V−I ratio [174a].

We extended the use of SIS for PT healing. After the central third of the tendon was harvested for ACL reconstruction, an SIS scaffold was introduced to aid its healing and limit the formation of adhesion (and permit motion) between the healing PT and the underlying fat pad (Fig. 67.7). The maintenance of stress and motion of the patella−femoral joint motion would keep the homeostasis of the remaining PT [63-65,72], thus limiting problems associated with poor healing, including excessive hypertrophy of the healing PT with poor quality.

When the SIS was applied to the anterior and posterior of a central-third PT defect (3 mm wide) in rabbits, the healing defect had a more organized matrix with a large number of spindle-shaped cells at 12 weeks after surgery compared with the untreated group, which had only patches of collagen with a sparse distribution of cells [174b]. The CSA increased by 61% in the SIS-treated group; concomitantly, the BPTB complex showed 38% higher stiffness and 58% higher ultimate load. These results clearly demonstrated that SIS treatment could accelerate PT healing.

Anterior Cruciate Ligament Healing

With the advances in FTE, alternative approaches are being revisited to healing the injured ACL. Investigators have employed various biological methods such as PRP, ECM bioscaffolds, and so on to incite and accelerate ACL healing. Meanwhile, investigators also recognize that the rate of ACL healing is slow, and thus mechanical augmentation could be advantageous to maintain knee stability to facilitate the biological processes. The following overview covers both biological and mechanical augmentation for ACL healing as well as the combination of biological and mechanical strategies.

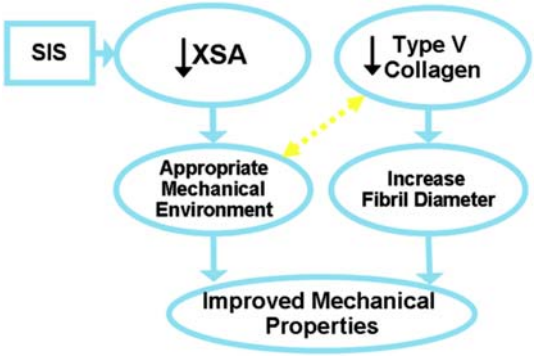

FIGURE 67.7 Schematic of possible mechanism of action of small intestinal submucosa (SIS) on medial collateral ligament healing response. *XSA*, cross-sectional area.

Biological Augmentation

There has been renewed interest in healing soft tissues with limited healing potential, such as the ACL, using biological augmentation. For example, Steadman and coworkers developed a microfracture technique that involves making very small holes in the bone at the femoral insertion site to encourage hematoma formation and provide an enriched environment for ACL healing [175,176]. This approach was used successfully to treat patients aged more than 40 years. In the laboratory, Murray and coworkers used collagen-PRP as a bioscaffold to heal a fully transected and sutured ACL in a porcine model [177,178]. At 4 weeks, the stiffness and load at yield and at failure were more than two times those of the suture-repaired control group [178].

In our research center, we used ECM bioscaffold to heal a surgically transected ACL in a goat model [84]. We combined SIS with SIS-hydrogel to heal a surgically transected ACL after primary repair. The SIS was derived from genetically modified Galα1-3Galβ−deficient pigs to reduce the potential immunological reaction in humans. The SIS sheet wrapped around the defect was used to contain the healing response at the injury site and guide tissue growth, whereas the SIS hydrogel was used to fill the defect and accelerate tissue formation by means of its desirable chemoattractant and angiogenic factors. With the new treatment, the transected ACL was found to heal with continuous neotissue by 12 weeks. Its CSA and shape were similar to those of the sham-operated intact ACL and were significantly more robust than the suture repaired (control) group. Morphologically, its collagen fibers were aligned with spindle-shaped fibroblasts. Functionally, the SIS-treated ACL had reduced anteroposterior knee instability compared with the control group whereas the in situ forces were similar to intact ACL. Furthermore, the structural properties of the FATC when tested in uniaxial tension showed that tensile stiffness was 2.5 times higher than suture repair controls and actually reached approximately 50% of the intact FATC.

A separate series of studies were performed by Nguyen and coworkers [179,180]. First attempts were made to suture the transected ACL and wrap it with SIS in a sheet form. Histologically, the collagen fibers were more dense and more compactly arranged than in the suture repair control group [180]. Afterward, an in vivo evaluation of this approach was performed and the CSA was found to be 50% of the intact ACL whereas stiffness for the SIS group was 35% higher than in the control group [179].

Studies have also shown, the feasibility of enhancing ACL graft integration after reconstruction using a triphasic scaffold using fibroblast and osteoblasts were seeded on a section of the scaffold that mimicked the native environment of the ligament insertion and bone, respectively [181,182]. Collectively these approaches demonstrate that ECM bioscaffolds have substantive potentials for improving treatment for injured ligaments and tendons.

Mechanical Augmentation

Mechanical augmentation has been shown to stabilize the knee with an injured ACL, which could aid in ACL healing. In a porcine model, Fleming and coworkers used sutures passing from bone to bone to reduce anterior joint laxity [183]. Our research center had also systematically used sutures (number 2 FiberWire sutures) for mechanical augmentation. In a goat model, we first determined the best locations of the bone tunnels for the sutures; i.e., the anterior footprint of the femoral origin and medial aspect of the tibial footprint on stifle joint stability after ACL transection [184]. In these positions, the anterior tibial translation was within 3 mm of the intact joint and the in situ force was similar to that of the intact ACL. A follow-up study showed the relative contribution of the soft tissues in resisting the anterior tibial load. Under the 67-N anterior tibial load, the ACL provided the dominant support in the intact

joint. In the case of ACL deficiency, the MCL and medial meniscus carried significant loads, reaching up to 36% and 53% of the intact ACL, respectively (at 30 degrees flexion). After suture repair of the ACL, the in situ force was 81% of the intact ACL whereas that for the augmentation sutures was 103% [185]. Thus, it could be concluded that suture augmentation provided good initial joint stability and concomitantly lowered the loads on the MCL as well as the medial meniscus.

Finally, an in vivo study was performed to examine whether suture augmentation could heal a surgically transected ACL in a goat model. After 12 weeks, the anteroposterior tibial translation of the stifle joint for the suture augmentation group was about 20% lower than that of the suture repair group (control) whereas the in situ force in the healing ACL was more than 50% higher. Morphologically, the ACL was found to heal with good neotissue formation. In terms of the structural properties of the FATC, the linear stiffness was 75% greater than that of the suture repair control. It was shown that suture augmentation had provided the needed stability to the stifle joint for intrinsic healing of the ACL to take place.

Combined Biological and Mechanical Augmentation

With evidence that biological and mechanical augmentation could individually enhance ACL healing, it begs the question whether their combination would work in synergy to enhance the ACL healing process further. It was hypothesized that mechanical augmentation would stabilize the knee immediately after surgery and enhance biological augmentation to stimulate and accelerate the ACL healing further.

For the purpose of mechanical augmentation, a novel Magnesium (Mg)-based ring was designed to bridge the two torn ends of the ACL and serve as an internal splint for mechanical augmentation (Fig. 67.8). Furthermore, the Mg ring could simultaneously load the healing ACL as well as its insertion sites to prevent disuse atrophy. The function of the Mg ring-repaired ACL was first evaluated in an in vitro study using a goat model. After the application of a Mg-based ring, the anterior tibial translation of the repaired ACL was reduced by 60−70% from the ACL-deficient state and was within approximately 3 mm of that of the intact stifle joint [186,187]. Mg-ring repair could also restore the in situ forces of the repaired ACL to within ±5 N of the levels of the intact ACL. These promising in vitro results suggest that the Mg-based ring is a good device for mechanical augmentation.

Then, the Mg-ring was used combined with ECM bioscaffolds in an in vivo animal study [186,187]. It was hypothesized that the Mg-based ring would degrade as the ACL healing progressed and its mechanical function would be replaced by the healing ACL, because Mg is biodegradable and bioresorbable [188]. After surgical transection, the Mg-based ring was sutured to connect the stumps of the ACL followed by wrapping the transection site with an ECM sheet and injection with ECM hydrogel (Fig. 67.8) [186,187]. After 6 weeks, the device had degraded 40%

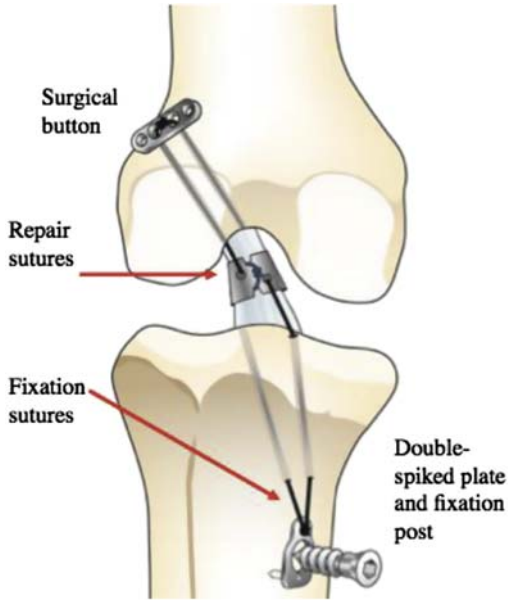

FIGURE 67.8 Schematic diagram showing the application of the Mg-based ring device to bridge the transected anterior cruciate ligament. Fixation sutures through the femoral bone tunnels are fixed using a surgical button whereas those through the tibial bone tunnels are fixed using a double-spiked plate and fixation post.

and translucent healing tissue was seen [186,187]. By 12 weeks, the function of the stifle joint was determined by the robotic–UFS testing system under 67 N anteroposterior load, the anteroposterior tibial translation of the Mg ring-repaired group was about 33% less than that of the suture repair group. The in situ forces carried by the Mg ring repair group were approximately twice those of the suture repair group. Similarly, the structural properties of the FATC (stiffness and ultimate load) for the Mg ring repair group were three times greater than the suture repair group [186,187]. These results also compared favorably to those after ACL reconstruction in the same animal model, because the stiffness was 1.5 times higher than that after ACL reconstruction in a goat model at 12 weeks [186,187,189]. Similarly, the ultimate load was 1.8 times higher. Thus, it is clear that the combined use of biological and mechanical augmentation therapy worked in synergy to accelerate ACL healing. Future work will include a long-term (26-week) study to determine whether these benefits persist.

SUMMARY AND FUTURE DIRECTIONS

In this review, biomechanical and biological problems facing repair and healing of ligament and tendon injuries were discussed. There have been tremendous improvements to clinical treatment paradigms based on studies that established a fundamental understanding of healing after ligament or tendon injury and the benefits of controlled mobilization. Nevertheless, many issues remain.

For ligaments and tendons that display healing potential after injury, major challenges are recovery of normal ultrastructural appearance, biochemical composition, and mechanical properties. Specifically, important steps to be taken are increasing the fibril diameters of healing tissues by limiting the production of type V collagen and decorin and improving the alignment of healing tissue by guiding the organization of newly produced matrix. By manipulating the healing response at the molecular and cellular levels and guiding tissue formation, the following FTE approaches may offer the potential to restore the properties of healing tissue to normal levels.

We are particularly interested in bioscaffolds such as ECM. Applied to a healing ligament or tendon in vivo, it serves as a substrate that provides contact guidance for cells to form more aligned collagen fibers with a concomitant improvement in mechanical and viscoelastic properties compared with untreated controls. Furthermore, the chemoattractant degradation products and bioactive agents of SIS could enhance the rate of healing [168], allowing better maintenance of stress and motion-dependent homeostasis. More excitingly, the SIS can be modified in vitro by seeding BMDCs on the scaffold and applying cyclic stretching to increase its alignment. Hence, when applied in vivo, the tissue engineered scaffold could serve to accelerate the initiation of the healing process by improving the production and orientation of collagen, which ultimately will help to make a better neoligament or tendon.

On the other hand, for ligaments and tendons that do not heal after injury and require surgical reconstruction using replacement grafts (e.g., ACL reconstruction), the major challenge is to promote a remodeling response so that the graft maintains sufficient stiffness and strength to provide functional stability of the joint. Most important is enhancing the rate of integration of tendon–bone interfaces during early graft incorporation, which may permit an earlier and more aggressive postoperative rehabilitation [190]. These complex issues may require a combination of approaches including gene and cell therapies as well as biologic scaffolds. Indeed, grafts treated with AdBMP-2 have shown some potential [137] in both canine and rabbit models. In addition, other biological tissues such as periosteum have been used to enhance the interface between tendon and bone, with some success [190]. All of these results suggest an exciting potential for clinical application.

Indeed, FTE has generated many exciting developments. For example, there is an exciting class of biodegradable metallic scaffolds, namely, porous magnesium or magnesium oxide, that has the advantage of initial stiffness to provide needed stability for the ligament to heal while performing its function. The degradation rate of these "smart" scaffolds could also be controlled as they are replaced by the neotissue. Furthermore, protein coating of these biodegradable metallic scaffolds could be performed for better tissue integration and controlled release of growth factors and cytokines to sustain tissue healing as well as guide tissue regeneration. In particular, Mg has been used combined with ECM bioscaffolds to heal the ACL, and it has the potential to serve as an alternative to surgical reconstruction.

To translate knowledge gained about a particular gene, protein, or cell to a clinical application will require expertise from many disciplines to work in seamlessly. One roles of biomedical engineers within this framework would be to link interactions of the functions of molecules to cells, cells to tissues, tissues to organs, and organs to body. When biologists, biomedical engineers, clinicians, and experts from other disciplines work together, this results in better therapies that lead to injured ligaments and tendons healing with properties closer to those of normal ligaments and tendons. Efforts of such a team-based approach to the new developments of FTE will bring a bright future to

the outcome of healing of ligaments and tendon injuries. With the development of precision medicine based on "big data" and cellular reprogramming on the horizon, it is easy to imagine that these FTE techniques for healing ligaments and tendons can be tailored to be patient specific to gain a more positive, long-term outcome.

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CHAPTER

68

Central Nervous System

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INTRODUCTION

The human central nervous system (CNS), which is composed of the brain, spinal cord, and retina, is a network of more than 100 billion individual nerve cells that control our actions, sense our surroundings, and define who we are. It has several unique anatomical and physiological characteristics, including enclosure in bony structures (skull and vertebrae) that provide protection yet also hinder access; separation from systemic blood circulation by endothelial cell tight junctions, which serve as a barrier to toxins yet prevent intravenous therapeutic delivery; and a limited capacity for self-repair, notwithstanding the presence of endogenous stem cells in the brain, spinal cord, and retina.

Injuries to the CNS include stroke, traumatic brain injury (TBI), spinal cord injury (SCI), and retinal degeneration. These conditions affect millions of people worldwide and cover the entire age spectrum. CNS injury in the pediatric population includes predominantly traumatic or congenital defects, whereas in an adult population the injuries are traumatic or degenerative. Worldwide, every year approximately 15 million people experience a stroke (World Heart Federation). The incidence of traumatic SCI is 180,000 per year globally [1] whereas the prevalence in North America is approximately 1 in 1500 people [2]. Retinal degenerative conditions such as age-related macular degeneration are projected to affect 196 million people globally by 2020 [3] whereas retinitis pigmentosa (RP), which has a pediatric onset, is the leading cause of inherited retinal degeneration-associated blindness [4]. For all of these conditions, acute and chronic populations pose different treatment challenges.

Neurons, the functional units of the CNS, transmit and store information; they are extremely vulnerable to injury and degeneration. Mature neurons are postmitotic cells incapable of cell division. Thus, lost cells cannot easily be replaced and often leave a patient with a permanent functional impairment. Furthermore, a significant challenge in CNS regeneration is the complexity of the injury site. The CNS contains multiple subtypes of neurons that form an intricate system of connections with surrounding cells, such as other neurons and glia, and a complex organization that is difficult to recapitulate with current repair strategies. The anatomical location of the injury also has a significant role in the extent of trauma; a parenchymal lesion of several cubic centimeters may be clinically silent: that is, unapparent to both patient and clinician (if in the frontal cortex), severely disabling (if in the spinal cord), or fatal (if in the brain stem) [5].

Despite the complexity of the CNS anatomy, physiology, and pathobiology, tremendous progress in tissue engineering approaches have been made for treatment. This chapter focuses on tissue engineering strategies aimed at achieving functional recovery after CNS injury, including those based on drug delivery, cell delivery, and biomaterial scaffolds. Commonalities in the response to injury and barriers to regeneration are reviewed as related to the tissues of the CNS. Then various therapeutic strategies under investigation for the treatment of CNS injuries and diseases are introduced, such as drug delivery and cell therapy. The second half of the chapter covers the application of regenerative medicine to three case studies in CNS pathology: stroke, SCI, and retinal degeneration.

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WOUND RESPONSE AND BARRIERS TO REGENERATION

The Blood–Brain Barrier

Under normal conditions, the CNS is considered to be immunoprivileged owing to the blood–brain barrier (BBB), which prevents entry of circulating immune cells and controls the influx of molecules into the brain. There are three main components of the BBB: (1) endothelial cells joined by tight junctions, which limit the paracellular flux of hydrophilic molecules; (2) astrocytic end feet, which tightly ensheathe the vessel walls and maintain the tight junctions; and (3) pericytes, which are important for structural integrity and tight junction formation. The BBB permits diffusion of dissolved gases such as O_2 and CO_2 along their concentration gradients. In contrast, the transport of larger molecules must be actively facilitated; nutrients (e.g., glucose) cross the BBB via transporters and endocytosis mediates the uptake of larger molecules (e.g., insulin) [6].

After injury, the BBB is damaged, leading to the initiation of an immune response cascade. Reperfusion with oxygenated blood results in the production of reactive oxygen species (ROS), which stimulate the secretion of inflammatory cytokines from ischemic cells. Activated inflammatory cells release matrix metalloproteinases (MMPs), which disrupt tight junctions between cells and the basal lamina and lead to the breakdown of the BBB. Breakdown of the BBB in turn causes leakage of plasma, red blood cells, and infiltration of immune cells such as neutrophils and other leukocytes that release proinflammatory factors [7,8]. The inflammatory response is initiated by peripherally derived immune cells such as macrophages and activated glial cells such as microglia that migrate into the injury site. Once there, macrophages and microglia release a wide range of soluble factors including cytokines, tumor necrosis factor, and interleukins (IL)-1, -6, and -10, as well as clear cellular debris by phagocytosis. The released cytokines induce the expression of secondary cytokines, chemokines, nitric oxide, and ROS, subsequently causing cell death [8a]. Pericytes of the BBB have been shown to have a role in scar formation in the spinal cord [9], which is discussed further in the following Reactive Astrocytes and the Glial Scar section.

Reactive Astrocytes and the Glial Scar

Under normal conditions, glial cells in the brain and spinal cord have a multitude of important roles, including maintenance and regulation of the extracellular microenvironment [10]. Astrocytes secrete numerous trophic factors that support neurons, regulate extracellular levels of ions and neurotransmitters, and provide an antioxidant defense for neurons [11]. They are also the main source of endogenous tissue plasminogen activator (tPA), an anticlotting agent, in the brain [12]. In the eye, the principal glial cells are the Müller glia, which support neuronal activity and integrity of the blood–retinal barrier (BRB) and protect retinal ganglion cells from glutamate and nitric oxide neurotoxicity [12a,13].

Injury to the CNS results in astrogliosis, the proliferation of glial cells that undergo morphological changes to become reactive astrocytes [14]. Reactive astrocytes have opposing roles in tissue repair: they are necessary for the wound response initially, yet inhibit regeneration. After stroke and SCI, reactive astrocytes and other cells produce proteoglycans such as chondroitin sulfate proteoglycans (CSPG) and myelin-associated glycoproteins (MAG) that inhibit axonal outgrowth [15]. Delivery of MMPs or chondroitinase ABC to the CNS after injury results in better cellular integration by degrading these inhibitory extracellular matrix (ECM) factors [16,17]. Despite the known detrimental effects of reactive astrocytes, it is clear that their role is more nuanced than previously thought. The ablation of reactive astrocytes after SCI exacerbates the severity of the injury [18] and has been shown to abolish axon regeneration in mice [19]. In the retina, Müller cell gliosis involves the upregulation of important intermediate filaments and the expression of neuroprotective cytokines and factors that encourage photoreceptor survival after injury [20]. Further work is needed to establish how to balance both roles of reactive astrocytes effectively to promote wound healing while facilitating axon regeneration.

Endogenous Stem Cells

Spontaneous host tissue regeneration depends on endogenous cells with the capacity to proliferate and differentiate. Although it was originally thought that repair of the CNS by endogenous mechanisms was unlikely caused by a lack of adult neurogenesis, this has been disproven. The two main regions that contribute to neurogenesis in the brain are the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone of the hippocampus [21–23]. Neural stem cells have also been found in the spinal cord, specifically in the ependymal layer of the subependymal zone of the central canal [24,25], as well as within the parenchyma after injury [25a]. After injury to the

spinal cord, these quiescent cells are stimulated to proliferate, resulting in cells positive for neural precursor markers (e.g., Sox2 and nestin) in the lesioned parenchyma [26]. Retinal stem cells have been identified in the pigmented ciliary epithelium of the eye; they proliferate in vitro and are differentiated into retinal-specific cell types in mice [27] and humans [28]. Astrocytes are another source of neurogenesis after injury; in the brain, it has been demonstrated that they are able to differentiate into functional neurons after stroke [29]. Although endogenous stem cells are stimulated by injury in the brain and spinal cord, they are not sufficient to induce recovery [30,31].

After CNS injury, a cascade of events ensues including BBB permeabilization, immune cell infiltration, and formation of a glial scar that inhibits the regeneration of tissue. Although endogenous mechanisms exist for protection (the glial scar) and repair (the proliferation of normally quiescent stem cell populations in response to injury), they are insufficient to result in significant functional repair on their own. Differences in wound response, anatomy, and cell type necessitate therapies tailored to the tissue target and pathological state, whether with biomolecule or cell-based therapeutics.

THERAPEUTIC STRATEGIES IN THE CENTRAL NERVOUS SYSTEM

Therapeutic strategies in the CNS can be classified as biomolecule or cell therapy. This section provides a broad overview of these approaches, acknowledging that the approaches are not exclusive to one another, and indeed most are typically complementary. Further detail is given in the case studies of CNS pathology (Section Case Studies in Tissue Therapy in the Central Nervous System).

Biomolecule Delivery

The use of biomolecule delivery as a protective and regenerative strategy after CNS injury has been investigated both in animal models and clinically. Molecules can be delivered systemically or injected locally. Systemic delivery is suitable only for molecules that can cross the BBB or blood–spinal cord barrier (BSCB) but the high doses required can result in undesirable side effects. Notwithstanding this limitation, many drugs acting on the CNS have been delivered systemically (for example, some small molecules such as methylprednisolone); however, molecules that do not cross the BBB, such as monoclonal antibodies, necessitate a local delivery strategy. Local injections can be performed into the epidural or intrathecal spaces of the spinal cord, into the ventricles in the brain, or directly into the tissue.

The goals of biomolecule delivery in the CNS are to limit degeneration (i.e., neuroprotection) and promote regeneration. Neuroprotection can be defined as a long-lasting maintenance of functional ability, as well as increased axonal sparing and reduced lesion volume. Neuroprotective molecules act on a variety of cellular targets, such as anti-apoptotic pathways to reduce death of certain neuronal populations or oligodendrocytes, and antiinflammatory pathways to inhibit microglial activation [32]. Growth factors previously explored for their regenerative potential have also been shown to be neuroprotective. For example, nerve growth factor (NGF), brain-derived neurotrophic growth factor (BDNF), neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF), and glial cell-line-derived neurotrophic factor (GDNF) all promote axonal elongation and enhance the survival of sensory and/or motor neurons [33–37].

Neuroregeneration includes the growth of existing axons, sprouting of new axons from neural cell soma, remyelination, and plasticity among surviving axons, all leading to tissue repair and functional recovery. Outgrowth of motor and/or sensory neurons has been reported with the use of neurotrophic factors such as NGF [38], BDNF [39], NT-3 [38], GDNF [34], CNTF [40], and both acidic and basic fibroblast growth factor (FGF-1 and FGF-2) [41], among others. Some factors are predicted to act in synergy with one another [42] and with a second class of neuroregenerative molecules, which target growth inhibitory glycoproteins and proteoglycans by activating the Rho–Rho-associated protein kinase (ROCK) pathway. For example, MAG and NogoA are native to the CNS and bind to the Nogo (NgR) and $p75^{NTR}$ cell surface receptors, thereby triggering Rho-ROCK and growth inhibition [43]. Blocking this interaction with anti-NogoA or anti-NgR allows neurite outgrowth to resume [44]. Rho activation (and growth inhibition) can also be inhibited by increased intracellular concentrations of the nucleotide cyclic-adenosine monophosphate (cAMP) [43] or Cethrin, which showed some success in a Phase I/II clinical trial [45]. Delivery of cAMP or Rolipram, an inhibitor of cAMP degradation, has also been shown to enhance neuroregeneration in animal models [46,47] but is not yet in clinical trials. Another approach to neuroregeneration is stimulation of endogenous stem and progenitor cells in the stem cell niche. For example, specific factors (e.g., epidermal growth factor [EGF], FGF-2, cyclosporin A (CsA), and erythropoietin [EPO]) delivered to the stem cell niche stimulate neural precursor cells to proliferate and migrate, supporting the replacement of cells lost owing to injury [48,49].

Cell Therapy

The goals of cell transplantation are to replace cells lost owing to injury directly and promote the survival of endogenous cells [50,51]. Stem cells ameliorate poststroke impairments in the brain by modulating inflammation, inducing angiogenesis, and secreting neuroprotective factors into host tissue [52–54]. Theoretically, the ideal cell type would meet three requirements: (1) be from a source that is simple to harvest and does not raise ethical concerns, (2) have the ability to proliferate and generate large populations of donor cells, and (3) be from an autologous source or a one that does not require immune suppression to minimize rejection of engrafted cells.

Despite some Phase I clinical success with mesenchymal stromal cells (MSCs) in stroke and SCI [55,56] embryonic stem cell (ESC)-derived oligodendrocyte progenitor cells (OPCs) in SCI [57], and retinal pigmented epithelium (RPE) cells in retinal degeneration (e.g., NCT01344993, NCT01469832), cell transplantation is still in its infancy and is often complicated by inconsistent results. A major challenge with transplanting allogeneic or xenogeneic cells is a lack of long-term transplant survival, likely the result of the hostile microenvironment of the injury [58–60]. Limited availability of allogeneic donor cells is also an issue; even committed neural precursors isolated from fetal tissue do not proliferate to the extent necessary for widespread clinical utility. An alternative strategy involves the directed differentiation of stem cells, which have the ability for unlimited growth in culture. Several stem cell sources have emerged with the potential for therapeutic application in the CNS: ESCs, adult neural stem cells, and adult nonneural stem cells such as MSCs and adipose-derived stem cells (ADSCs). In addition, induction of pluripotent cells or transdifferentiation from adult somatic cells allows for the generation of patient-specific tissue grafts derived from the patient's own cells.

ESCs are pluripotent cells isolated from the inner cell mass of preimplantation blastocysts. The inner mass cells proliferate and differentiate to become the cells that make up the three germ layers of developing humans with the potential to become any cell in the body (pluripotent). This propagation potential can be recapitulated in culture and differentiation of these cells can be directed in a lineage-specific manner to yield terminally differentiated cells [61]. Safety is an important consideration in the clinical application of ESCs. Although there are concerns that their proliferative ability may lead to tumor and mass formation [62], none have been reported clinically. Predifferentiation of these cells is a means to diminish the implied risk of directly transplanting pluripotent cells [63]. However, in the absence of directed differentiation toward a particular cell fate, impure populations of differentiated cells can arise [64]; therefore, ESCs and their progeny should also be purified to ensure that only cells of a neural lineage are transplanted [64a]. The difficulty of translating human ESC (hESC) therapies to the clinic is highlighted through work done with hESC-derived cells for SCI by the California-based company, Geron. In early 2009, the US Food and Drug Administration (FDA) approved the first clinical trial for an hESC product, in which a suspension containing hESC-derived OPCs was injected directly into the SCI lesion site [65]. This trial was suspended twice in the first year: once because of questions regarding the purity of the cell population and then owing to microscopic cysts in the regenerating injury site in animal models. Geron halted the project completely in 2011 [66]. In 2014, the clinical trial resumed under the direction of Asterias Biotherapeutics [67], which after reviewing the preclinical safety of the OPCs [57] reported that the first eight patients showed promising results and that a further five to eight patients were to be enrolled.

Neural stem-progenitor cells (NSPCs) reside in distinct regions of both the fetal and adult CNS, namely the subventricular zone of the lateral ventricles [21,22], subgranular zone of the dentate gyrus of the hippocampus [68,69], and ependymal layer of the spinal cord central canal [24,25]. These cells undergo proliferation and maturation throughout adult life [70]. NSPCs can be isolated from embryonic or adult CNS and expanded in culture. These cells have been shown to survive, differentiate into all classes of cells in the CNS, integrate, repair tissue, and improve neurological function after transplantation into animal models of CNS disease or injury [71,72]. Adult NSPCs have lower survival than fetal NSCs after transplantation in the injured brain [73,74], and most transplanted cells differentiate into astrocytes in vivo rather than neurons or oligodendrocytes [75,76].

Nonneuronal stem cells are those derived from other tissues of the body and include MSCs, hematopoietic stem cells, and ADSCs. The majority of clinical stem cell transplantation trials to treat ischemic stroke, as listed by the National Institutes of Health clinical trial registry, use a nonneural source of cells such as autologous bone marrow–derived MSCs or hematopoietic stem cells. There are two major advantages to using these cells: (1) they are easily obtained from the body in large quantities, and (2) they can be derived from an autologous source, which eliminates immunogenicity [77]. MSC transplantation in rodents results in increased functional recovery, whereas their autologous transplantation in patients showed no adverse effects [77a,78]. It is thought that the regenerative success seen with MSCs results from their ability to secrete a variety of neuroprotective factors, such as BDNF, into the injury site to support endogenous cell survival and regeneration [79]. Work on them includes genetically engineering MSCs to overexpress certain factors that will further enhance their therapeutic effect [80].

The derivation of induced pluripotent stem (iPS) cells [81] from dermal fibroblasts has made it possible to harvest adult terminally differentiated cells and revert them to an ESC-like state [82]. Early iPS cell methods involved infection of the cells with a viral vector to induce pluripotency; current methods such as use of the *piggyBac* transposon allows the transgene to be excised once pluripotency is established, avoiding the risk of mutagenicity caused by a permanently integrated factor [83]. Exploration of this approach may eventually lead to the development of patient-specific (autologous) neural grafts grown from the patient's own cells.

The skin also contains a special type of precursor cell, termed "skips" [84,85], which has shown promise for the treatment of neurological disorders [86] such as SCI [87,88].

Transplanted cells have a high rate of cell death because of a variety of factors including proapoptotic signals, the immune response, and anoikis, that is, the lack of a scaffold for cell adhesion [58–60]. The relatively poor survival of transplanted cells suggests that a combination of cells and drug/biomolecule delivery would enhance survival and integration of the transplanted cell population. Combining delivery of neural stem or progenitor cells with chondroitinase ABC and growth factors enhanced cellular integration and functional recovery in a rat model of chronic SCI [89]. Chondroitinase ABC improves the host environment for cell engraftment by degrading the inhibitory CSPGs in the glial scar, promoting cell integration, whereas the growth factors EGF, FGF-2, and platelet-derived growth factor (PDGF)-AA enhance stem cell survival and differentiation [90–92]. To support cell survival and provide an extracellular framework for the transplanted cells, an engineered composite vehicle that can deliver both the cells and biomolecules to the therapeutic target in the CNS is desirable [93,94,94a].

CASE STUDIES IN TISSUE THERAPY IN THE CENTRAL NERVOUS SYSTEM

Stroke

Cerebrovascular disease, defined as an abnormality of the brain caused by a pathological process involving its blood supply [95], is the third leading cause of death in the United States. In terms of morbidity and mortality, it is one of the most common neurological disorders. There are two main types of stroke: hemorrhagic, caused by a ruptured cerebral blood vessel, and ischemic, caused by an occlusion in the cerebral vasculature. Approximately 87% of strokes are ischemic [96]; thus, we focus on ischemic stroke.

Pharmacological Therapy

Pharmacological treatment of stroke can be broadly classified into two categories, thrombolytic and neuroprotective. The goal of thrombolysis is to remove the blood clot that results in ischemia so that normal blood flow to the brain can be restored. However, there is an increased risk of hemorrhagic complications with this treatment and it is imperative to administer it within hours after the initial occlusion for it to be effective [97]. Thrombolysis treatment promotes the conversion of the proenzyme plasminogen in the bloodstream into the active enzyme plasmin, a protease that degrades blood plasma proteins associated with blood clotting, most notably fibrin. The only FDA-approved treatment for stroke is tPA, which activates plasminogen by catalyzing its conversion into plasmin and improves the outcome in ischemic stroke when administered up to 4.5 h after stroke [98].

In the hours and days after a stroke, there is continued cell death and impaired function in the penumbra, the area surrounding the lesion core. The penumbra contains cells that are nonfunctioning but can be rescued using therapeutic strategies [7]. Neuroprotective treatments focus on preventing further damage and preserving as much tissue as possible. Neuroprotective agents being investigated include calcium antagonists, glutamate antagonists, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid antagonists, opioid antagonists, γ-aminobutyric acid (GABA) agonists, free radical scavengers, antiinflammatory agents, and membrane stabilizers [99]. A particularly compelling example of a successfully translated neuroprotective agent is NA-1, an inhibitor of NMDA receptor-mediated neuronal death, which has been shown to reduce infarct volume in both primates [100] and humans [101] and is being tested in a US-based Phase II trial [102].

Factors for Endogenous Stem Cell Stimulation

After stroke, neural stem cells within the SVZ express elevated levels of receptors for growth factors. For example, receptors for both GDNF [103] and EGF [104] are increased. This suggests that the injured brain may be receptive to repair by its endogenous stem cell populations through growth factor delivery. In animal models of stroke, administration of EGF and EPO stimulated endogenous stem cells, resulting in regeneration of damaged tissue after pial vessel disruption [105]. Sequential administration of β-human chorionic gonadotropin (hCG) and EPO 24 h after

stroke resulted in a significant decrease in lesion volume and functional improvement in an animal model [106]. Moreover, a clinical study demonstrated that a similar sequential administration of intramuscular β-hCG and intravenous EPO 24–48 h after stroke is safe and resulted in a trend toward smaller infarct size [107]. Granulocyte-colony stimulating factor (G-CSF) has also shown promising results: Cells in the SVZ express the receptor for G-CSF and subcutaneous administration of this factor stimulated stem cell proliferation, resulting in improved functional recovery [108].

An alternative to stimulating the endogenous brain stem cells is to target bone marrow–derived stem cells, which will home to the site of injury. When G-CSF was administered intravenously to rats subjected to middle cerebral artery occlusion (MCAO), infarct volume decreased, suggesting a neuroprotective effect [109]. Moreover, a clinical study showed that subcutaneous administration of G-CSF is a safe and feasible method to mobilize bone marrow stem cells [108].

CsA, a clinically used immunosuppressant, has also been shown to stimulate neural stem cells in the SVZ [110]. CsA, encapsulated in poly(lactic-*co*-glycolic acid) (PLGA) microspheres and delivered locally to the injured cortex, demonstrated increased cell proliferation in the SVZ and a smaller infarct volume compared with untreated controls [48] (Fig. 68.1). Work has also been done to investigate the use of BDNF for stroke recovery. BDNF levels in the brain

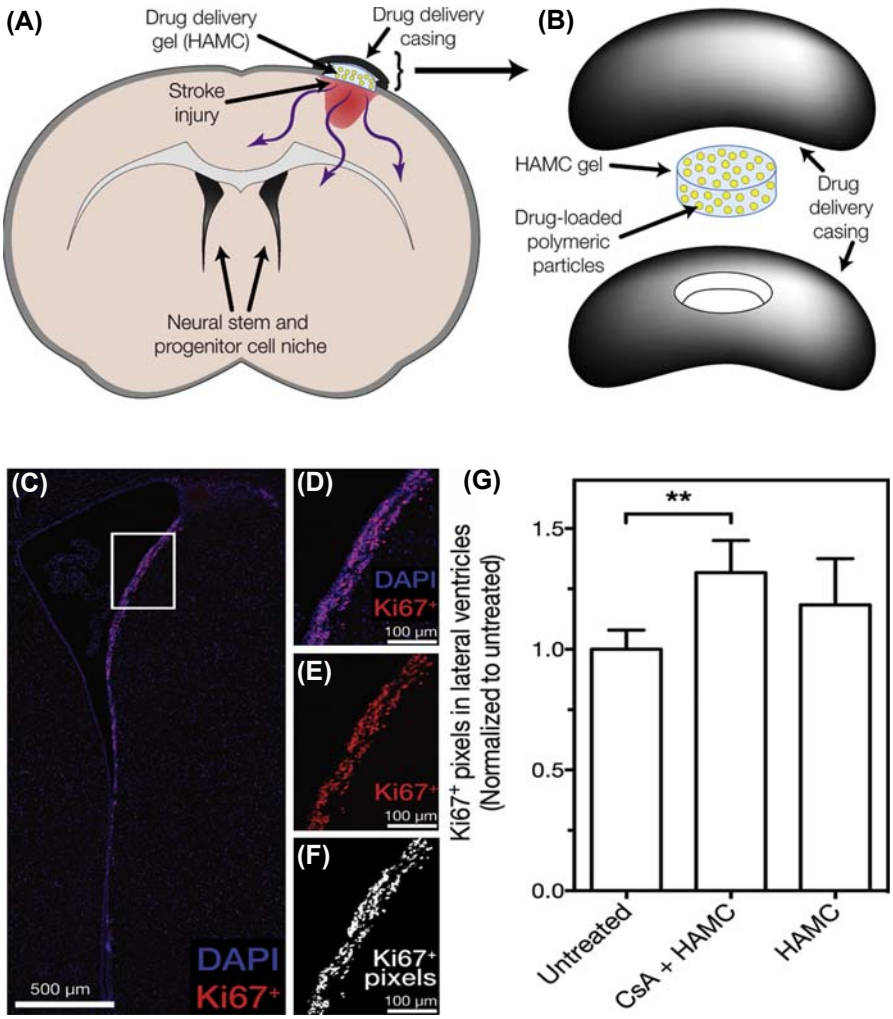

FIGURE 68.1 A localized drug delivery system for the delivery of cyclosporin A (CsA) to the stroke-injured rat brain. (A, B) A hydrogel (hyaluronan and methylcellulose [HAMC]) containing drug-loaded polymeric microspheres is injected onto the surface of the brain and contained in a polycarbonate casing. (C–G) CsA delivery to the injured brain results in a significant increase in Ki67-positive proliferative cells in the subventricular zone compared with untreated animals. *DAPI*, 4′,6-diamidino-2-phenylindole. Magnification, 20X. *Reprinted with permission from Tuladhar A, Morshead CM, Shoichet MS. Circumventing the blood-brain barrier: local delivery of cyclosporine A stimulates stem cells in stroke-injured rat brain. J Control Release 2015;215:1–11.*

are elevated after injury and it is important for poststroke recovery [111,112]. Delivery of BDNF, either systemically or locally, results in reduced infarct volume and improved functional recovery [113–115].

An exciting alternative to drug delivery is the delivery of viral gene vectors such as NeuroD1 to influence the direct in vivo reprogramming of reactive astrocytes of the glial scar into new neurons that can integrate into the neural circuity. A study demonstrated that this is possible in the cortex of injured and Alzheimer model mice, in which NeuroD1 was used to reprogram astrocytes successfully into glutamatergic neurons [116]. This strategy avoids the issue of immune rejection of cell transplants and may be able to generate large enough numbers of endogenous neurons while depleting the glial scar for functional recovery. Further work is needed to increase transfection efficiency and generate the neuronal subtypes needed for circuit restoration [117].

Cell Transplantation

Because of limited endogenous regeneration in the brain, exogenous cells have been transplanted as an alternative strategy. When NSPCs were administered intravenously to mice subjected to MCAO, the cells migrated to the injured side of the brain; however, only 0.09% of the injected cells were found in the brain whereas many were detected in other organs [52]. Local delivery strategies include transplantation directly into the lesion or into the surrounding penumbra, and can achieve greater numbers of cells at the injury site than systemic delivery. Directly into the lesion is favored because it is able to accommodate the injected volume without damaging healthy tissue and is adjacent to the penumbra, a region of potentially recoverable cells [93,93a]. However, despite these benefits, the hostile inflammatory environment of the lesion is not always conducive to the survival of transplants, which is limited to 1–10% of the injected population [58,118]. Because of the varying size of lesions, an injectable hydrogel that fills the irregular void is desirable (Fig. 68.2).

To support cell survival and provide an extracellular framework for cell delivery, scaffolds are being explored to promote greater cell survival and regeneration [93,94a]. Biomaterials can act as three-dimensional artificial niches to support cellular function and survival, provide physical protection from washout into the cerebrospinal fluid (CSF), and provide protection from the immune response and apoptotic signals from the ECM [58–60,60a]. Natural scaffolds such as Matrigel have been used to deliver neural progenitor cells (NPCs) into the stroke lesion and showed an increased number of cells at the transplantation site relative to delivery in artificial CSF [120]. Notwithstanding these results, Matrigel is derived from a mouse sarcoma and is inherently ill-defined, which hinders its clinical use. To elucidate whether individual components of Matrigel can improve cell survival, cells have been transplanted in collagen I [121] and a mixture of collagen I and either laminin or fibronectin [122], both of which were found to improve cell survival. Another natural polymer that has garnered much attention is hyaluronan (HA). HA constitutes a significant proportion of the CNS ECM and can be cross-linked to have a modulus similar to that of native brain tissue [60a,93]. HA is also reported to inhibit scar formation and promote angiogenesis in vivo [123,124].

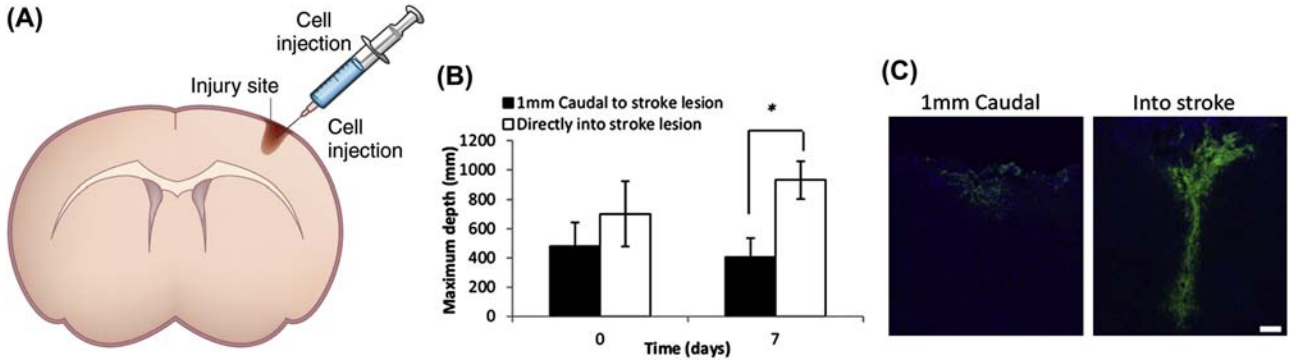

FIGURE 68.2 Injectable delivery vehicle for cell delivery to the injured brain. (A) This conceptual drawing shows injection of the space-filling vehicle into the cystic cavity formed after traumatic or stroke-induced brain damage. A craniectomy is performed to expose the injury site and a hydrogel is then injected to fill the cavity. (B, C) Comparison of injection of mouse neural stem/progenitor cells directly into the mouse stroke lesion (*white bars*) or 1 mm caudal to the lesion (*black bars*). At 7 days after injection, cells delivered directly into the lesion achieved greater depth into the brain than those delivered caudal to the lesion. Scale bar, 100μm. *(A) Reprinted with permission from Reichert W. Indwelling neural implants: strategies for contending with the in vivo environment, vol. xviii. Boca Raton: CRC Press; 2008. p. 271. (B and C) Reprinted with permission from Ballios BG, Cooke MJ, Donaldson L, Coles BLK, Morshead CM, van der Kooy D, Shoichet MS. A hyaluronan-based injectable hydrogel improves the survival and integration of stem cell progeny following transplantation. Stem Cell Rep 2015;4(6):1031–45.*

Combined with methylcellulose, HA methylcellulose (HAMC) hydrogels increased cell survival and motor recovery when used as a vehicle to deliver neural stem cells (NSCs) into the stroke-injured cortex of adult mice [94].

When stem cells are delivered to the site of injury, they must differentiate into the correct cell types for integration and functional recovery. It has been reported that later-stage neuronal precursors from the embryonic cortex are better able to differentiate and survive when transplanted into the stroke-injured brain compared with cells of an earlier developmental stage [73]. Similar results were observed with transplanted human iPS-derived neuronal precursors [125] and ESC-derived neuronal precursors [126]. Specific regions of the brain or different microenvironments have also been shown to drive stem cells to differentiate preferentially into glia over neurons. For example, adult NSCs from the SVG or SVZ will produce neurons only when they are transplanted in neurogenic regions; in nonneurogenic areas they differentiate primarily into glial cells [75,76,127]. An experiment comparing the transplantation of glial-restricted spinal cord progenitors and NSCs into the aging hippocampus reported that regardless of initial cell type, cells differentiated into predominantly glia, which suggests that the microenvironment of the hippocampus influences preferential differentiation of stem cells into astrocytes [128]. This phenomenon is also observed in the spinal cord, where it has been reported that if cells are not restricted to a neuronal lineage before transplantation, they will mainly differentiate into glia and exhibit lower survival [129].

A combination therapy using growth factors or peptides and stem cells has been proposed to better control and improve the efficacy of cell transplantation. For example, the combined delivery of EPO and MSCs results in increased proliferation of cells along the lateral ventricle wall and greater functional recovery than either EPO or MSC treatment alone [130]. Combining cells with peptides has a similar outcome; NSCs delivered with the adhesion peptide IKVAV, derived from the full-length laminin protein, to a TBI rat model increased neuronal cell attachment and neurite outgrowth only when IKVAV was present [131].

Rehabilitation can contribute to regeneration when combined with stem cell transplantation. To study the effects of rehabilitation, animals are housed in enriched environments (EE) where they are allowed access to a variety of objects that stimulate exploratory behavior in addition to free access to individual running wheels [132]. Combined with mouse NSPC transplantation, EE enhanced functional recovery and proliferation of endogenous progenitor cells and increased cell migration to the injury site [132]. In contrast, when rats were injected with neural precursor cells derived from human ES cells, EE had no significant effect on functional recovery [58]. The authors attributed these varying results to possible differences in the immune response and cell survival between mouse and human stem cells.

Preclinical studies demonstrate the potential of stem cell therapy to treat stroke; however, to translate this potential to the clinic, additional research is required to control cell survival, differentiation, and integration better for ultimate functional repair. Although thrombosis treatment has had the best clinical success, the short window for treatment limits its utility. Ultimately, combination strategies that both protect brain tissue from further degeneration and promote tissue regeneration will likely see the greatest success in the clinic.

Traumatic Spinal Cord Injury

Traumatic SCI results from contusion/compression or transection (laceration) of the cord. Compression injuries comprise 70% of clinical cases and partial or complete transection comprise the remaining 30% [133]. Compression of the spinal cord may be caused by dislocation of a vertebra and pinching of the cord between the anterior and posterior faces of the vertebral foramen in adjacent vertebrae. In spinal cord trauma, the level and severity of cord injury determine the extent of neurological manifestations and the prognosis. Damage to thoracic and lower vertebrae can lead to paraplegia and cervical injury may result in quadriplegia. Spinal cord injuries above the fourth cervical vertebra can also compromise respiratory function. Segmental damage to the ascending and descending white matter tracts accounts for the principal clinical deficits.

The initial primary injury causes uncontrolled necrotic cell death, inflammation, ischemia, and hypoxia. These processes persist for weeks and initiate a second wave of apoptosis in neurons and oligodendrocytes, increasing the volume of injured tissue and forming a spindle-shaped cystic cavity [134]. This secondary biochemical cascade is also evident after transection, although a cavity does not form. In both injury types, the injured tissue is isolated from the environment by reactive astrocytes through the formation of a glial scar [134]. Similar to stroke, tissue therapy after SCI is classified as neuroprotective (reducing the biochemical damage of the secondary injury) or neuroregenerative (restoring lost function through regrowth and reinnervation of axons).

Biomolecule Delivery

Classes of biomolecules used for spinal cord repair include steroidal compounds, sodium or potassium channel blockers, and growth factors. The steroid methylprednisolone, an antiinflammatory drug, is used clinically [135], but

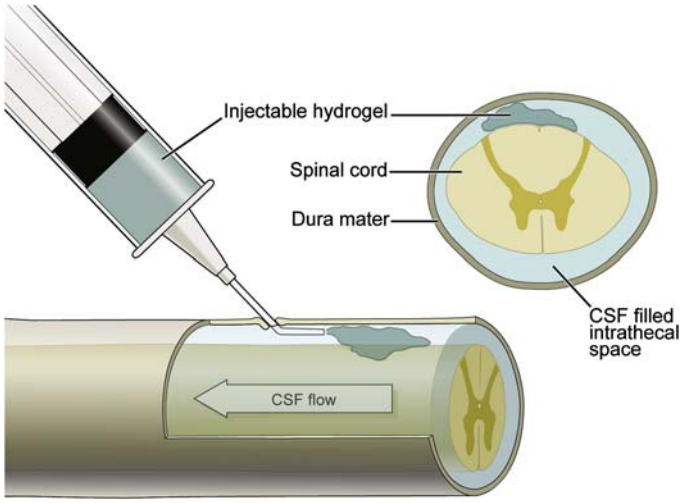

FIGURE 68.3 When injected into the intrathecal space, a hydrogel-based drug delivery system can localize and modulate biomolecule therapeutic release at the site of injury. This route is preferred over epidural delivery, in which the diffusive barrier presented by the dura mater is significant. *CSF*, cerebrospinal fluid. *Copyright (2005) by Michael Corrin, reproduced with permission of the artist.*

its beneficial effects have been questioned and it is not always administered to patients. Minocycline, a tetracycline derivative, targets the host inflammatory response and can cross the BSCB [136]. It demonstrated modest motor improvements in a Phase II trial [137], which is recruiting for a Phase III trial [138]. Riluzole, a sodium channel blocker in clinical use to treat amyotrophic lateral sclerosis, was tested in a Phase I SCI trial, which was completed in 2013; and is currently undergoing a Phase 2/3 trial (ClinicalTrials.gov identifier NCT01597518; [138a]).

Because most drugs and protein therapeutics delivered systemically cannot cross the BSCB, the most effective delivery strategies to the injured spinal cord are to the intrathecal space or directly into the cord. Local release circumvents the BSCB and enables direct tissue benefit. For example, anti-NogoA (ATI355, Novartis) was injected directly into the intrathecal space using an external pump or catheter. Although the Novartis trial results have not been published, use of the external pump caused infections in the first patients, resulting in a change of strategy to bolus injection to the intrathecal space [139]. While not circumventing the BSCB, Cethrin (Vertex) was delivered locally to the epidural space from a fibrin sealant [140]. The Cethrin trial resulted in modest improvement in cervical spinal cord–injured patients 12 months after treatment [45]. Despite these results, with epidural delivery the therapeutic molecule must cross the dura mater, arachnoid mater, and fluid-filled intrathecal space before reaching the damaged spinal cord, resulting in limited tissue penetration.

Intrathecal injection is a desirable strategy for local delivery because it bypasses the two meninges of epidural delivery and avoids both the infection associated with external pump or catheter and the tissue damage associated with the needle track of intraspinal delivery (Fig. 68.3). Continuous intrathecal infusion is most often achieved from an indwelling catheter and pump system. Implanted osmotic minipumps have been used in many preclinical in vivo studies for up to 28 days [44,141,142]. Continuous infusion provides excellent dose control, but prolonged catheterization carries with it the risk for tissue damage by catheter tips that can compress and scar the spinal cord [143]. Injectable, bioresorbable hydrogels provide an excellent alternative to the pump or catheter system. Prolonged release for up to 2 months has been demonstrated with a series of protein and drug therapeutics. For example, bolus injections of sustained release formulations, such as drug-loaded polymer particles, distribute throughout the intrathecal space [144] and hydrogels in which polymeric nanospheres are incorporated remain localized at the site of injection, achieving prolonged release. HAMC was injected into the intrathecal space in a rat model of SCI, where it remained localized for up to 7 days [145]. Drug-loaded PLGA nanoparticles were included in the HAMC formulation to sustain drug release and stabilize the hydrogel, resulting in HAMC composites that were stable for more than 50 days and sustained drug release for 28 days in vitro [146]. This formulation provided a platform for the local delivery of factors (NT-3 and anti-NogoA [90]; and cell–drug combinations such as PDGF and neural stem cells [92], or the combination of PDGF, a Gly–Arg–Asp–Gly–Ser adhesive peptide, and OPCs [91] to the injured spinal cord.

Guiding Axon Regrowth

A challenge particular to transection injuries of the spinal cord is the long-distance axon regeneration required for functional recovery. Thus, treatment strategies for transection injury use physical constructs to provide a permissive

environment and guidance cues for axonal regeneration. When the cord is completely severed, the stumps can be placed within nerve guidance channels in a strategy analogous to that pursued in peripheral nerve repair. Tube dimensions, wall thickness, porosity, and mechanical strength are all important physical factors that influence the suitability of tubes for CNS repair. Matching the mechanical properties, and specifically the modulus, of the tube to the tissue has been shown to be important to avoid necrotic tissue at the interface [147]. Considerable effort has been directed into optimizing tube fabrication methods, including polymer extrusion, casting in molds, immersion of a polymer solution-coated mandrel in a nonsolvent, and centrifugal casting of phase-separated polymerization mixtures. These techniques vary in the uniformity of the tubes produced; centrifugal casting remains the most versatile option for hydrogels and molding is the simplest [148,149]. Tubes are fabricated from a variety of both synthetic and natural materials to meet these criteria increasingly to function as platforms for drug and/or cell delivery.

One of the most popular classes of materials is hydrogels such as poly(2-hydroxyethyl methacrylate-*co*-methyl methacrylate) (P[HEMA/MMA]) [150]. P(HEMA/MMA) is biocompatible and is promising as a nerve guidance tube in the CNS and peripheral nervous system [148,149]; however, P(HEMA/MMA) is nondegradable and investigators have turned to materials such as poly(L-lactide) [151], poly(hydroxybutyrate) [152], chitosan [153,154], and collagen [155], which are degradable in vivo. Hydrogels alone have been used when the cord is partially or fully transected to fill the tissue defect and promote bridging of the gap [156,157]. The alignment of the material is also an important consideration for conduit design; it has been demonstrated that unilateral nanogrooves aligned with the direction of axon growth result in better directional guidance than less-organized topographic cues within the conduit [158].

Various cell types have been incorporated into physical constructs to enhance axon guidance and survival in the spinal cord. Schwann cells [151,152,159], astrocytes [159], and NSPCs [160,161] have been included in the inner lumen of tubes, either adherent to the inner surface or suspended in a hydrogel within the tube. Cell-adhesive hydrogels such as dilute collagen [162] and laminin-functionalized agarose [163] have shown promise in this application. One example involves rat NSPCs that were seeded in fibrin scaffolds inside chitosan channels and combined with dibutyrylcyclic adenosine monophosphate–releasing PLGA microspheres to promote recovery in the rat [163a].

Cell Transplantation

Similar to stroke, cell delivery to the injured spinal cord is challenging owing to the hostile microenvironment, glial scarring, and a lack of ECM. Nonetheless, there is significant interest in intraspinal cell delivery and investigators have used many of the cell types previously mentioned for this purpose. In acute injury models, cells have been injected into the tissue as a suspension in buffer [164,165] or polymer matrix [166] near the site of injury. One challenge unique to spinal cord regeneration is the remyelination of spared axons, thought to be an important mechanism for recovery. Predifferentiation in vitro of human ES cells to oligodendrocyte progenitors, before transplantation, has been demonstrated to increase remyelination after transplantation compared with undifferentiated cells [64a,167]. Differentiation of hESCs to oligodendrocyte progenitor cells is the basis of a clinical trial led by Asterias, as mentioned earlier. In another study, NSCs derived from either a myelinating (wild-type) or nonmyelinating (*Shiverer* mouse) cell line showed that although both cell types integrated and predominantly differentiated into oligodendrocytes, only animals that received myelinating cells showed tissue and functional benefits [168] (Fig. 68.4). The use of cells that are predifferentiated to a specific type of the *neuronal* lineage, such as GABAergic interneurons, has also shown success in restoring function and reducing neuropathic pain after SCI [169,170]. Interestingly, robust corticospinal tract regeneration in a complete transection rat model was observed after transplantation of NPCs driven toward a spinal cord identity, which suggest that the positional identity of progenitor cells is important for integration into the injury site [171].

Retinal Degeneration

In the normal eye, light reaches the neural retina, which is composed of photoreceptors (rods and cones) and is transduced into electrical impulses for the brain by various cell types (Fig. 68.5). The retinal pigment epithelium (RPE), located directly adjacent to the photoreceptors, supports photoreceptor metabolic function and has a critical role in the biochemical process of vision. Retinal disease leads to permanent loss of visual function for which there is no definitive treatment. Vision is a major factor in quality of life; in the United States, it is estimated that $4.6 billion dollars is spent on non–diabetes related retinal disorders (Prevent Blindness America). The search for cures is made more urgent by the knowledge that as populations in the developed world age, the incidence of blindness caused by

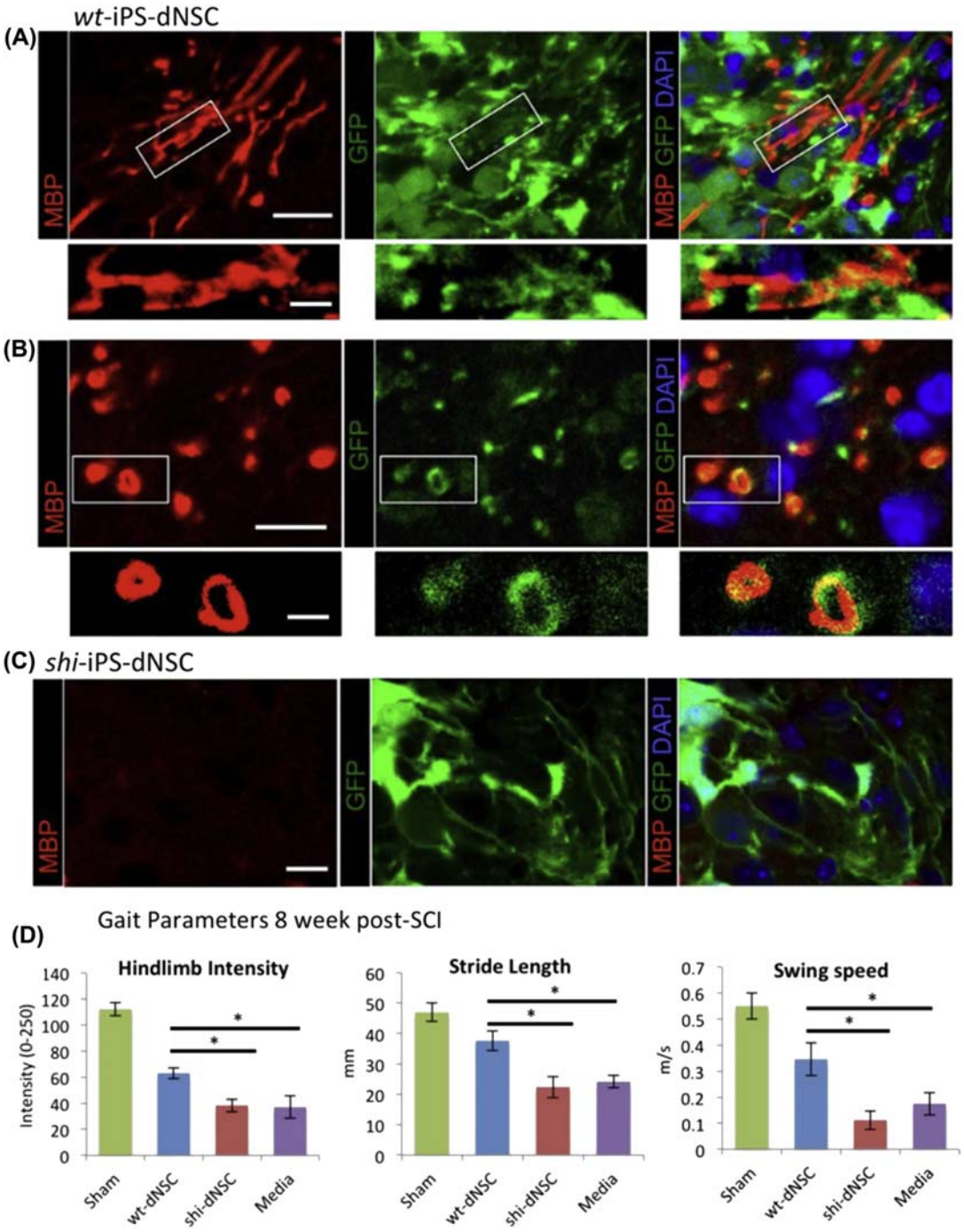

FIGURE 68.4 Remyelination is important to recovery in spinal cord injury. The delivery of induced pluripotent stem cell-derived neural stem cells (iPS-dNSC) into the injured spinal cord from wild-type (*wt*) mice (A, B) results in significant behavioral recovery attributed to increased myelination compared with iPS-dNSCs from the *shiverer* (*shi*) mutation. (C) After transplantation, the wild-type iPS-dNSCs showed remyelination myelin basic protein (MBP plus staining) of axons in both longitudinal and transverse orientations, whereas the *shiverer* iPS-dNSCs showed no myelination. (D) Gait parameters at 8 weeks after injury were significantly improved in only the wild-type dNSC group. *DAPI*, 4′,6-diamidino-2-phenylindole; *GFP*, green fluorescence protein. *Reprinted with permission from Salewski RP, Mithcell RA, Li L, Shen C, Milekovskaia M, Nagy A, Fehlings MG. Transplantation of induced pluripotent stem cell-derived neural stem cells mediate functional recovery following thoracic spinal cord injury through remyelination of axons. Stem Cells Transl Med 2015;4(7):743–54.*

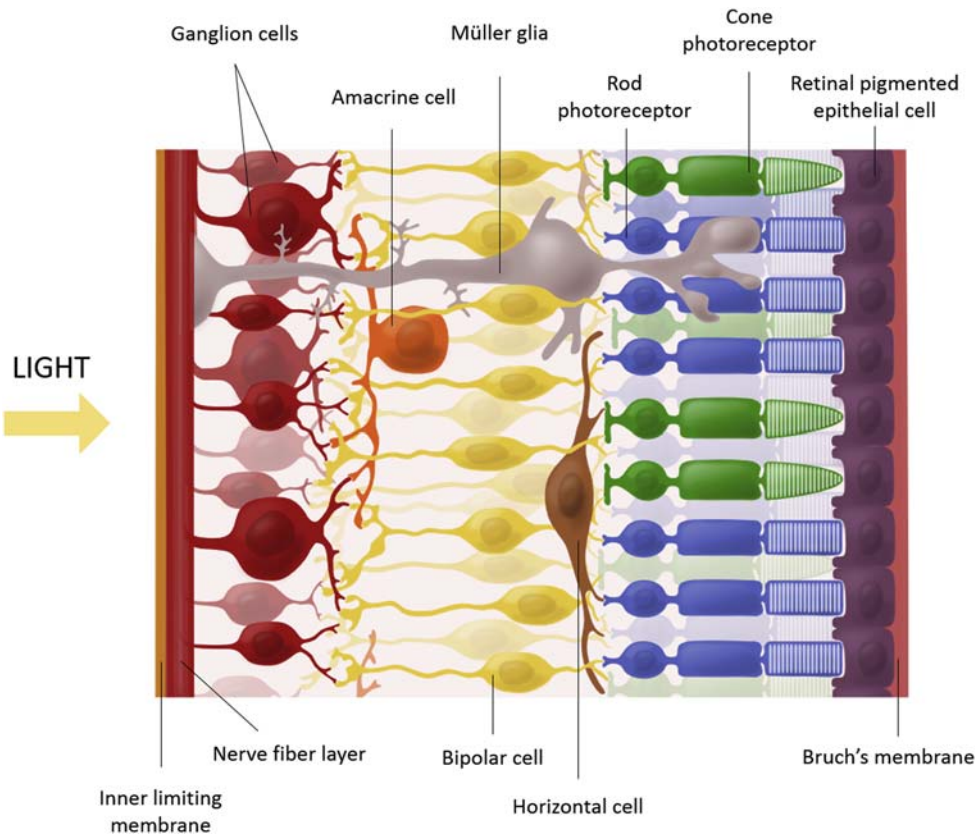

FIGURE 68.5 The human retina possesses a laminar structure. Photoreceptors (rods and cones) lie in the outer nuclear layer, interneurons (bipolar, horizontal, and amacrine cells) in the inner nuclear layer, and ganglion cells in the ganglion cell layer. Photoreceptors, bipolar cells, and horizontal cells make connections with each other in the outer plexiform layer. Bipolar, amacrine, and ganglion cells make connections in the inner plexiform layer. Information flows from outer lamina to inner lamina, as well as laterally via horizontal and amacrine cells. *Reprinted with permission of Delplace V, Payne SL, Shoichet MS. 2015. Delivery strategies for treatment of age-related ocular diseases: from a biological understanding to biomaterial solutions. Journal of Controlled Release. 219: 652–68.*

to retinal degeneration is expected to rise [171a,172]. Age-related macular degeneration (AMD) is the most common cause of irreversible vision loss in the United States [173]. To understand regenerative therapies for retinal degeneration, it is important to understand the pathogenesis of a typical degenerative condition such as AMD. It is commonplace to describe AMD as "dry" (atrophic) or "wet" (exudative). Dry AMD is identified by diffuse, discrete deposits in the Bruch membrane (drusen) and atrophy of the RPE, whereas wet AMD is characterized by choroidal neovascular membranes originating from the underlying choriocapillaries. These vessels are leaky and exudate can form macular scars that directly destroy both photoreceptors and the RPE. The RPE is essential for homeostatic photoreceptor outer segment phagocytosis and disruption of the RPE is associated with photoreceptor death [174,175].

Biomolecule Delivery

Retinal degeneration therapies are focused on pharmacotherapy. These therapies slow disease progression but do not restore vision loss. There have been significant advances in the treatment of wet AMD with antivascular endothelial growth factor (anti-VEGF) therapies [176,177]. Examples of available biomolecules include ranibizumab (Lucentis), bevacizumab (Avastin) [178–180], and pegaptanib (Macugen). One issue with anti-VEGF treatment for the eye is the serious off-target effects on VEGF-dependent physiological processes such as wound healing and kidney function. To address this, an anti-VEGF "sticky-trap" composed of anti-VEGF and a heparin-binding domain was designed to remain at the site of injection and thus avoid systemic effects [181]. Other experimental treatments in AMD and diabetic retinopathy focus on eliminating oxidative damage using bioactive molecules such as advanced glycosylation end-product inhibitors and antioxidants [182]. Corticosteroids and antiinflammatory drugs such as dexamethasone have been explored extensively; a number are available commercially [183,184]. An interesting example is Ozurdex, a degradable implant composed of PLGA-encapsulated

dexamethasone used to decrease macular edema in patients with diabetic retinopathy [184]. Gene therapies might also present a method for targeting defects that result from inherited retinal disorders such as Leber congenital amaurosis [185], RP [186], and ocular albinism [187].

Cell Transplantation

Cell transplantation is an alternate strategy that arguably, has seen the most clinical success in retinal degeneration compared with other tissues of the CNS. Early clinical studies conducted in patients with retinal degeneration involved retinal sheet transplants. This procedure relies on an immature retinal sheet taken from fetal tissue to extend processes and integrate with the degenerating recipient retina. The rationale is that spared inner cells of the retina require only synaptic connections with functional photoreceptors to complete the visual circuit. Various types of tissue have been allografted: fetal RPE cells in patients with AMD [188] and neural retinal cells in patients with RP [189]. These studies have shown some subjective improvement in function but also eventual rejection of the transplant [190–192]. Some of the most promising results to date were reported with the transplantation of retinal progenitor cell (RPC) sheets with intact RPE into 10 patients with RP and AMD, in which seven patients showed some improvement in visual acuity scores without cell rejection [193].

Studies suggested that stem cell transplantation shows promise for reconstituting damaged cell populations in the retina [194–196] (Fig. 68.6). Preclinical research on cell transplantation is directed toward transplanting cell suspensions into the subretinal space, and strategies involving the delivery of NPCs, healthy photoreceptors,

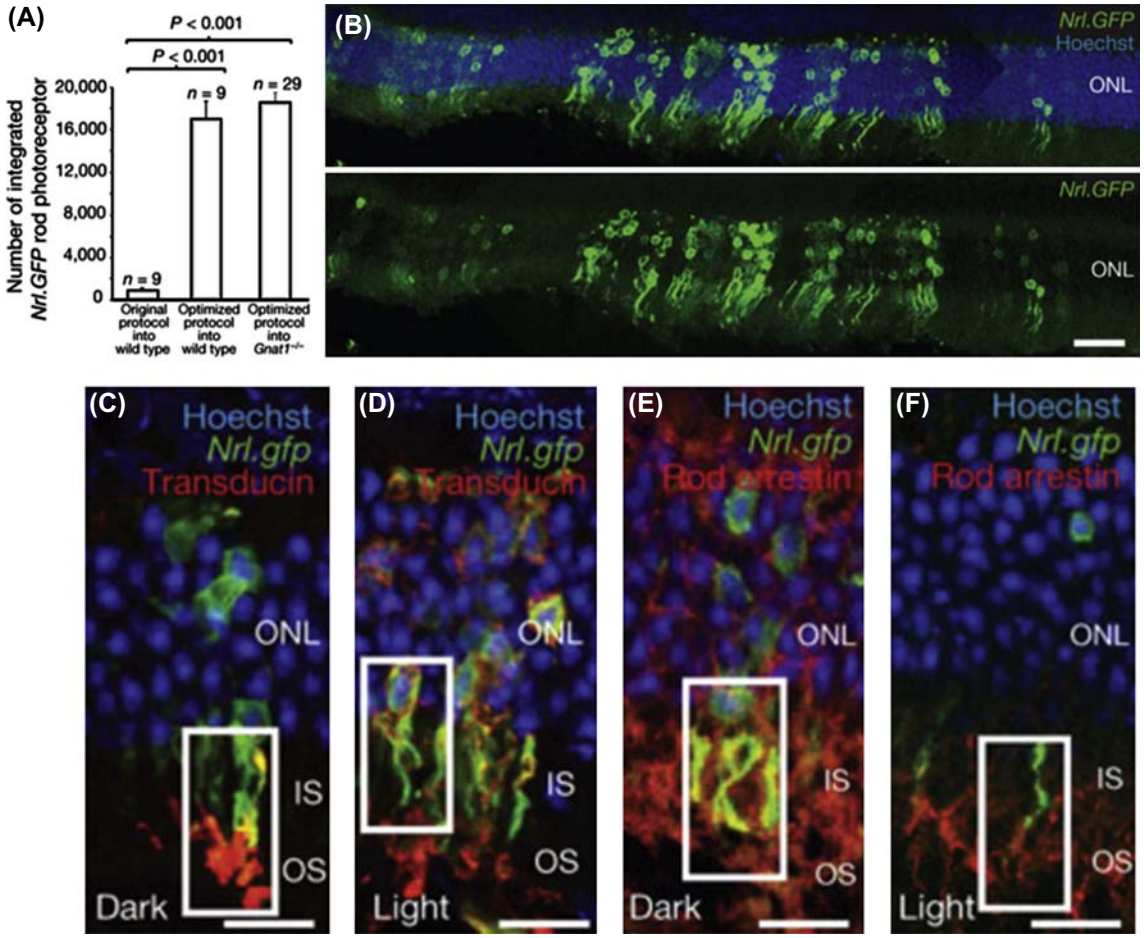

FIGURE 68.6 *Nrl*.GFP (neural retina leucine zipper gene; green fluorescent protein) rod transplantation results in integration into the adult *Gnat1*$^{-/-}$ retinal dysfunction mouse model. (A) Optimization of transplant protocol led to an increase in the number of photoreceptors present after transplantation. (B) Representative images of integrated photoreceptors (green fluorescence protein positive) in the retina with and without 4′,6-diamidino-2-phenylindole staining. Scale bar, 50μm. (C–F) Integrated photoreceptors expressed appropriate proteins (transducin and rod arrestin) at the correct location during light and dark stimulation. Scale bar, 10μm. *IS*, inner segment; *ONL*, outer nuclear layer; *OS*, outer segment. *Reproduced with permission from Pearson RA, Barber AC, Rizzi M, Hippert C, Xue T, West EL, et al. Restoration of vision after transplantation of photoreceptors. Nature 2012;485:99–103.*

photoreceptor precursors, and RPE cells are all being pursued. Immature neurons are capable of migrating and differentiating during neural development, and many studies have focused on the integration of these brain-derived neural progenitors into the retina. However, transplantation to the adult retina showed limited donor cell integration [197,198]. It was assumed that the adult retina lacks the extrinsic cues that are present during development to aid donor cell integration [199]. The inability of neural precursors to differentiate into photoreceptors suggested that a more appropriate cell source for transplantation might be neural RPCs. These cells, which are from the developing embryonic retina, are native to the retinal microenvironment and may generate photoreceptors more readily [196,200]. Morphological differentiation and integration have been observed with transplanted RPCs into the retina of adult pigs [201]. The Ali Lab demonstrated the integration of functional photoreceptors into the retina of adult mice, but only when the donor cells were postmitotic photoreceptor precursors [195,202]. These transplants showed functional synaptic activity and the ability to recover light response when transplanted into *rho*$^{-/-}$ mice, a model of selective photoreceptor loss, and *Gnat*$^{-/-}$ mice, a model lacking rod function. These studies suggest that the ontogenetic stage of the transplanted cell is likely crucial for successful integration into the outer nuclear layer of the retina and association with other photoreceptors. Interestingly, transplantation of more mature photoreceptors resulted in higher cell survival than immature RPC progeny [94,202,203]. An unexpected phenomenon, in which transplanted photoreceptors exchange intracellular material, such as green fluorescent protein, with host cells has been reported. This results in false-positive results when quantifying cell integration. This important finding may necessitate a reinterpretation of the mechanism of transplant-mediated functional recovery [204–206].

Efforts using pluripotent cell sources have focused on deriving a purified cell population for transplantation. Various protocols have been developed for enriching the proportion of ESCs that differentiate along a retinal lineage [207–209]. These cells show the ability to integrate into models of retinal degeneration [208,210] and restore some visual function in models of photoreceptor dystrophism [207]. However, the absence of a method to purify these populations means a mixed population of differentiated cells is transplanted [63]. A major challenge is in vitro amplification of adult stem cell populations to yield sufficient cell numbers for clinical utility [211]. van der Kooy reported isolation of adult retinal stem cells (RSCs) from both the mouse eye [27] and human eye [28]. These multipotent cells can differentiate to all retinal cell types and are able to survive, migrate, and integrate into the developing neural retina. A major limitation of these cells is that photoreceptors make up only a small proportion (<10%) of differentiated cells in vitro. Stable transfection of genes known to be important in determining photoreceptor fate has greatly increased the number of photoreceptors obtained under differentiation conditions and shown integration of functional photoreceptors after transplantation into developing retina [212]. In addition, the use of exogenous factors for directing the differentiation of RSCs toward rod photoreceptor fate has allowed pure populations of donor photoreceptors to be generated for transplantation without the need for gene transfection [213]. Although progress has been made in generating and transplanting stem cell-derived rod photoreceptors, little has been achieved to address cone photoreceptor loss. Given the importance of cone photoreceptors for light vision, this is an area of active research. The Wallace group has reported a protocol for cone enrichment and demonstrated that transplantation into the retina results in integration and morphological maturation of the cones [214].

Used as a transplant model, the developing mouse host eye is permissive to donor cell integration but the adult retina presents unique challenges for integration. Cell death, leakage, and migration from the transplantation site occur when cells are delivered as a suspension in saline [196]. To overcome these barriers in the adult, interdisciplinary studies including the combination of regular tissue culture with tissue engineering are being pursued. For example, retinal progenitors isolated during development are grown and transplanted on solid biomaterial scaffolds [215–217]. Although these reports are an important advancement, the scaffolds employed do not match the modulus of the retina and may lack the flexibility required for subretinal delivery [217]. More recent advances for RPE delivery include the synthesis of synthetic membranes that more closely match the physical properties of the inner collagenous layer of Bruch membrane, the location of the native RPE [218,219]. These membranes improved the survival of human RPE in the rabbit retina after transplantation [220]. Materials such as synthetic polymers and collagen have been explored for this application with successes in animal models of AMD [220,221]. An alternative to implantable scaffolds is injectable vehicles such as an in situ gelling, bioresorbable hydrogel-based cell delivery vehicle for transplanting the adult RSCs into the subretinal space of adult mice [94,222]. This hydrogel allowed for normal RSC survival and proliferation and for greater continuous integration of RSCs into the RPE versus saline vehicle controls [94]. Hyaluronan-based hydrogel scaffolds were also shown to promote the survival of primary photoreceptors in vitro [223]. This cell delivery strategy may be useful for treating advanced maculopathy, in which large areas of RPE are destroyed [215]. These advances in solid and injectable scaffolds bring a new dimension to the treatment of retinal disease in which the scaffold itself can provide a prosurvival and prointegration environment for transplanted cells based on its composition. Translation to the clinic will depend on further increasing cell survival and host tissue integration to restore visual function.

CONCLUSIONS AND OUTLOOK

Regenerative therapy in the treatment of CNS injury is at the forefront of techniques that address the source of functional deficits. Important advances have been made in many disorders including stroke, SCI, and retinal degeneration. The combination of drug delivery, cell delivery, and biomaterials scaffolds is a promising approach to create a permissive environment populated with cells primed for regeneration. For this reason, future efforts will continue to see a merging of strategies of cells and biological factors with biomaterials-based scaffolds important for cell survival and integration, two key limitations of cell transplantation today. Continued innovation is required in the field of scaffold design to provide the optimal microenvironment for cell survival, differentiation, and integration. The biochemical and mechanical properties of the scaffold, in addition to cell−cell interactions, are active areas of research in biomimetic scaffold design.

Cell delivery requires determining the optimum population for transplantation that produces significant integration with existing neuronal circuitry and functional recovery. Optogenetics, a tool that allows for the precise control of the action potential of a transplanted neuron using light, can elucidate the mechanisms behind functional recovery after cell transplantation. Controlling the firing of transplanted cells after delivery and integration into tissue may provide direct evidence of whether cells are truly integrating into the host circuitry or relying on the secretion of factors, and which mechanism is more important for functional recovery [224,225].

For endogenous stem cell stimulation, advances in drug delivery technology will allow temporal and spatial control of released factors; yet, more work is needed to determine which factors to release and when. Moreover, for local release, new delivery strategies will be required that are minimally invasive and ensure diffusion through CNS tissue, thus encouraging local stimulation of stem cells. Because many cells have common receptors, uniquely stimulating the endogenous stem cells among other resident cells in a tissue may be difficult. The complexity of the CNS and its numerous protective barriers has made the intricacies of its function difficult to understand and repair. Notwithstanding these challenges, the promise of regenerative medicine in the CNS can build on successes in other tissues and advance treatments to the clinic.

List of Acronyms and Abbreviations

aCSF Artificial cerebrospinal fluid
ADSC Adipose-derived stem cell
ALS Amyotrophic lateral sclerosis
AMD Age-related macular degeneration
AMPA α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB Blood−brain barrier
BDNF Brain-derived neurotrophic growth factor
BRB Blood−retinal barrier
BSCB Blood−spinal cord barrier
cAMP Cyclic-adenosine monophosphate
CNS Central nervous system
CNTF Ciliary neurotrophic factor
CsA Cyclosporin A
CSF Cerebrospinal fluid
CSPG Chondroitin sulfate proteoglycan
dbcAMP Dibutyrylcyclic adenosine monophosphate
ECM Extracellular matrix
EE Enriched environments
EGF Epidermal growth factor
EPO Erythropoietin
ESC Embryonic stem cell
FGF-1 Fibroblast growth factor-1
GABA γ-aminobutyric acid
G-CSF Granulocyte-colony stimulating factor
GDNF Glial cell-line-derived neurotrophic factor
GFP Green fluorescent protein
HA Hyaluronan
HAMC Hyaluronic acid and methylcellulose
hCG Human chorionic gonadotropin
IL Interleukin
iPS Induced pluripotent stem
MAG Myelin-associated glycoprotein

MCAO Middle cerebral artery occlusion
MMP Matrix metalloproteinase
MSC Mesenchymal stromal cells
NDMA N-methyl-D-aspartate
NGF Nerve growth factor
NgR Nogo receptor
NPC Neural progenitor cell
NSC Neural stem cell
NSPC Neural stem-progenitor cell
NT-3 Neurotrophin-3
P(HEMA/MMA) Poly(2-hydroxyethyl methacrylate-*co*-methyl methacrylate)
PDGF Platelet-derived growth factor
PLGA Poly(lactic-*co*-glycolic acid)
ROS Reactive oxygen species
RP Retinitis pigmentosa
RPC Retinal progenitor cell
RPE Retinal pigment epithelium
RSC Retinal stem cell
SCI Spinal cord injury
SGZ Subgranular zone
SVZ Subventricular zone
TBI Traumatic brain injury
TNF Tumor necrosis factor
tPA Tissue plasminogen activator
VEGF vascular endothelial growth factor

Acknowledgments

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CHAPTER

69

Peripheral Nerve Regeneration

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PROBLEMS AND CHALLENGES WITH PERIPHERAL NERVE INJURIES

Injuries to the peripheral nervous system (PNS) occur frequently and are a major source of disabilities. PNS injuries impair the ability to move muscles, feel normal sensations, and can lead to painful neuropathies. PNS injuries are classified as being traumatic, nontraumatic, or surgical in nature. Traumatic nerve injuries result from collisions, motor vehicle accidents, gunshot wounds, fractures, lacerations, or other forms of penetrating trauma. Each year around 360,000 patients experienced traumatic peripheral nerve injuries in the United States. Only 15% of the patients had PNS injuries that could be treated, and around 50% of them showed no measurable signs of recovery or had drastically reduced muscle strength. Nontraumatic peripheral nerve injuries are mostly attributed to nerve compression and adhesion. It is estimated that approximately 400,000 repair procedures are performed in the United States each year to correct carpal tunnel syndrome, a nontraumatic nerve injury [1]. Although treatments for nontraumatic nerve injuries have higher efficacy than do traumatic nerve injuries, patients still face pain, loss of muscle strength, and reduced dexterity for several weeks to months. Surgical nerve injuries result from medical procedures such as prostatectomy. Prostatectomy procedures most often require sacrificing one or both cavernosal nerves, which adversely affects erectile function and bladder control. In this chapter, we review several techniques used to repair peripheral nerve injuries and discuss current limitations that need to be overcome in order to enhance functional recovery after nerve injury.

HISTORICAL BACKGROUND

The simplest technique for nerve repair in the case of nerve transection injuries is coaptation of the two ends of the nerve using sutures or fibrin glue. However, in many cases there might be a loss of nerve segment owing to injury or there might be a time lag between the injury and surgical repair during which the nerve ends might retract, resulting in a nerve gap. In such cases, end-to-end nerve suturing cannot be done without creating tension in the nerve segment, resulting in a poor regeneration outcome [2]. To overcome this problem, the two nerve ends are approximated using grafts, such as nerve autografts or allografts, muscle grafts, vein grafts, muscle–vein grafts, and synthetic nerve guidance conduits or nerve guidance channels (NGCs).

Historically, during the 19th and early 20th centuries, various materials were used to promote nerve repair, such as bone, metal tubes, blood vessels, and fat sheaths [3–6]. The use of autologous nerve grafts was also first reported during this time [7]. However, because of improper surgical techniques, anatomical repair rarely led to an appreciable return of function. During World War II, surges in numbers of nerve injuries led to advances in microsurgical techniques and instrumentation. Further refinements in microsurgical techniques and drug therapies have had beneficial effects [8]. Significant advances in surgical techniques have since been achieved and biological factors rather than surgical techniques limit improvements in nerve regeneration. Further advances may come from a greater understanding of the molecular mechanisms of nerve regeneration. Advances in multiple areas such as

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nerve cell culture, biomaterials design, and genetic techniques may lead us to develop NGCs that can successfully repair injured nerves.

CURRENT STRATEGIES FOR PERIPHERAL NERVE REGENERATION

Nerve autografts are considered the reference standard for bridging nerve gaps in the PNS [9]. Autologous tissue grafts possess several advantages such as low immunogenicity and a structural support that promotes cell adhesion and migration [10]. However, there are several disadvantages to this technique. Obtaining a natural graft could lead to loss of function and potential neuroma formation at the donor site, multiple small grafts may be needed in case of a long nerve gap, and there could be a size mismatch between the donor nerve graft and the injured nerve. More important, complete functional recovery is seldom obtained with autografts [11]. Therefore, there is interest in developing treatments and biomaterials that match or exceed the functional performance of autografts.

Natural or synthetic scaffolds, known as NGCs, for peripheral nerve regeneration consist of a tubular structure that serves as a "guidance channel" to direct nerve regeneration from the proximal to the distal end. In addition, scaffolds contain multiple properties or elements inside the tubular structure that promote and guide nerve regeneration, reduce scar tissue formation, and reduce inflammation, among others. In general, scaffolds for nerve repair should support axonal proliferation, have low antigenicity, support vascularization, be porous for oxygen diffusion, and avoid long-term compression. The use of NGCs reduces tension at the suture line, protects regenerating axons from the infiltrating scar tissue, and directs the sprouting axons toward their distal targets. The luminal space of NGCs can be filled with growth-promoting matrix, growth factors, and/or appropriate cells. In some cases of nerve repair, NGCs have been used to leave a small gap intentionally between the injured nerve ends to allow the accumulation of cytokines, growth factors, and cells [12]. Also, the NGCs can be used as an excellent experimental tool to control the distance precisely between the nerve stumps, test the fluid and tissue entering the channel, and vary the properties of the channel. Overall, NGCs can be thought of as having four central components that contribute to peripheral nerve regeneration: (1) scaffold/substrate, (2) growth factors, (3) extracellular matrix (ECM) molecules, and (4) cells. A nerve graft may have a combination of these components or all four of them. In this analytical framework, the presence of these components and their spatiotemporal distribution determine the efficacy of the graft (Fig. 69.1). In this chapter, the grafts are classified as isotropic or anisotropic based on the distribution of these four central components. In isotropic grafts, the components are distributed uniformly within the graft with no directional cues. In anisotropic grafts, one or more of these components is distributed nonuniformly to create a gradient that may provide a directional cue, usually to direct the axonal growth toward the distal target.

ISOTROPIC SCAFFOLDS FOR NERVE REGENERATION

Natural Scaffolds

Isotropic natural materials used as scaffolds include veins, skeletal muscle fibers, and collagen. Although these materials support nerve regeneration, they do not provide directional cues to the axons. Autologous vein grafts have been shown to provide a good environment for axonal regeneration in short nerve gaps [13,14]. However, the use of vein grafts for long nerve gaps has been less successful because of the collapse of veins caused by their thin walls and constriction owing to the surrounding scar tissue [15]. To prevent vein grafts from collapsing and improve their performance, intraluminal space fillers such as autologous Schwann cells (SCs), collagen, and muscle fibers have been used. Collagen-filled vein grafts were found to promote better axonal growth than empty vein grafts for a 15-mm nerve gap in rabbits [16,17]. Similarly, SC-seeded venous grafts supported axonal growth and performed better than did unseeded grafts to repair 40- and 60-mm nerve gaps in rabbits [18,19]. The principal drawback of this approach is that it requires the availability of the relevant amount of live autologous SCs (up to 8 million cells/mL), which are difficult to obtain. Muscle–vein combined grafts, in which the muscle fibers are inserted in veins, were used in 10-mm-long nerve gaps in rats and were found to promote axonal regeneration comparable to that of syngeneic nerve grafts [20]. Although the muscle–vein grafts were able to promote nerve regeneration in 55-mm-long nerve defects in rabbits, they were not comparable to nerve autografts [20]. Autologous muscle–vein combined grafts have been used clinically in humans to bridge nerve gaps ranging from 5 to 60 mm. The results were scored as "poor," "satisfactory," "good," and "very good," based on the recovery of sensory and motor functions. Of the 21 lesions repaired (in 20 patients), 10 were of the sensory nerves and 11 were mixed nerve lesions. All lesions in

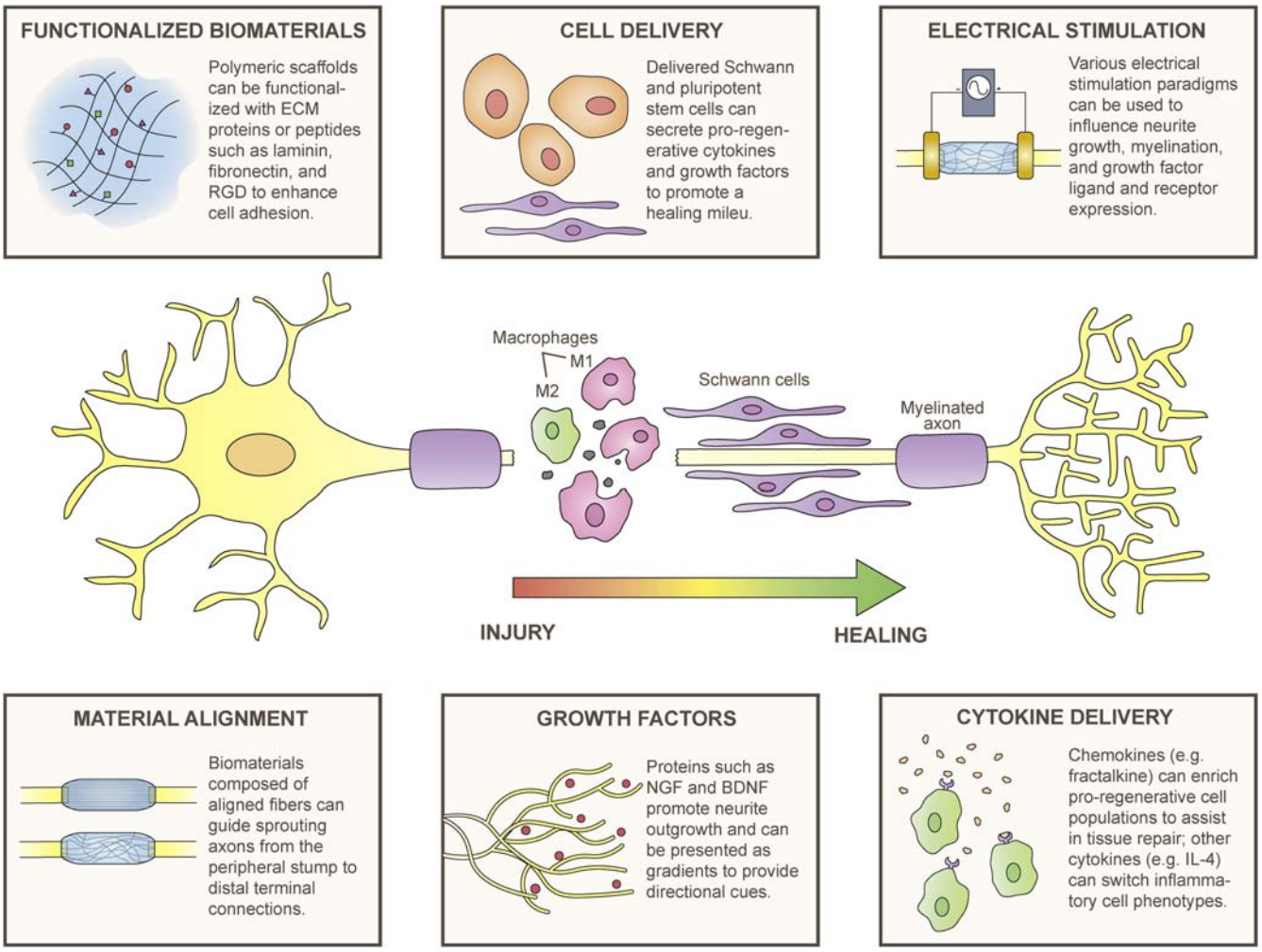

FIGURE 69.1 **Strategies for peripheral nerve regeneration.** Research in this field has encompassed multiple strategies, each targeting a unique aspect of the healing process after nerve injury. Future iterations in the design of scaffolds could combine these strategies and possibly evoke a synergistic response in nerve repair and regeneration. *BDNF*, brain-derived neurotrophic factor; *ECM*, extracellular matrix; *IL-4*, interleukin 4; *M*, macrophage; *NGF*, nerve growth factor; *RGD*, Arg-Gly-Asp. *Image courtesy of Dr. Syed Faaiz Enam, Duke University.*

the sensory nerves except one greater than 30 mm showed "good" to "very good" recovery. All lesions in the mixed nerves showed "satisfactory" to "good" recovery of motor and sensory functions [21]. Although autogenous/natural materials have shown encouraging results when used for nerve repair, they have certain drawbacks. In the case of autogenous grafts, drawbacks include the need for a second surgery, loss of function at the donor site, and neuropathic pain at the donor site. Allografts have problems related to preservation and immune rejection. To circumvent these problems, researchers have developed artificial or synthetic scaffolds for peripheral nerve.

Synthetic Scaffolds for Nerve Repair

Unlike natural scaffolds, synthetic scaffolds can be tailored to augment nerve regeneration in terms of their mechanical, chemical, and structural properties (Fig. 69.1). Nerve regeneration in silicone NGCs has been studied in detail [22]. Within a few hours of nerve repair using an NGC, the tube fills with serum exuded by the cut blood vessels in the nerve ends. This fluid contains neurotrophic factors as well as several cytokines and inflammatory cells such as macrophages. The macrophages remove the myelin and axonal debris formed as a result of injury. The fluid also contains the clot-forming protein fibrin. Within days, the fibrin coalesces and forms a longitudinally oriented fibrin cable bridging the two nerve ends. Without the formation of the fibrin cable, axonal regeneration cannot occur, which makes the fibrin cable formation a critical step. The fibrin cable is then invaded by cells migrating from the proximal and distal nerve stumps, including fibroblasts, macrophages, SCs, and endothelial cells (which form

capillaries and larger vessels). Axons from the proximal end grow into the fibrin matrix and are engulfed in the cytoplasm of SCs. Some of these axons then reach the distal nerve end and become myelinated. In inert silicone tubes of 10 mm or shorter, these processes occur spontaneously. However, it is generally accepted that impermeable, inert NGCs such as silicone do not support regeneration across defects larger than 10 mm without the presence of exogenous growth factors. The regeneration process can be improved by various approaches such as changing properties of the tube (permeability, porosity, texture, and electric charge characteristics) adding matrices, neurotrophic factors, ECM molecules, and cells [23]. These strategies augment nerve regeneration by affecting the sequence of events that lead to bridging of the nerve gap.

Synthetic NGCs can be classified as impermeable, semipermeable, and resorbable based on the porosity and/or degradability of the material used. Silicone tubes are an example of an impermeable NGC because they do not permit the movement of molecules across tube walls. Porosity affects the movement of soluble factors, oxygen, and waste products into and out of the NGCs, which is vital for nerve regeneration. Examples of semipermeable tubes are polysulfone and polyacrylonitrile/polyvinylchloride (PAN/PVC). Nerves regenerated in semipermeable tubes featured more myelinated axons and less connective tissue [24,25]. PAN/PVC channels with a molecular weight cutoff of 50 kDa support regeneration even in the absence of a distal nerve stump [24]. Examples of bioresorbable tubes are polylactic acid (PLA), polyglycolic acid (PGA), poly(L-lactide-*co*-glycolide) (PLGA), poly(lactide-*co*-caprolactone), and poly(3-hydroxybutyrate). The use of bioresorbable tubes negates the need for a second surgery to remove the implant and prevents long-term compression of the nerve. However, it is critical that degradation of the tube not allow fibroblasts to invade the lumen space before regeneration occurs, because this may prevent axons from regenerating.

Hydrogel Scaffolds for Nerve Repair

NGCs can be filled with gels to support axonal elongation. Here we briefly describe some of the isotropic gels used for nerve regeneration. Agarose is a polysaccharide derived from red agar and is widely used in gel electrophoresis and gel chromatography. SeaPrep agarose hydrogel has been shown to support neurite extension from a variety of neurons in a nonimmunogenic manner [26–28]. Agarose gels also allow molecules to be covalently linked to the gels through functional groups on their polysaccharide chains. For example, laminin protein or fragments of laminin can be covalently coupled to SeaPrep agarose gels to enhance their ability to support neurite extension [29]. Although agarose gels support neurite growth on their own, the coupling of molecules such as laminin significantly enhances the gels' ability to promote neurite extension.

Collagen is another gel commonly used to promote PNS regeneration. Collagen can be used to fill the intraluminal space of a vein graft to prevent it from collapsing and improve its nerve repair efficiency. In collagen-filled vein grafts, the number and diameter of myelinated axons were significantly increased compared with vein grafts without collagen gel [16,17]. Nerve repair with silicone tubes has also been significantly improved by filling them with collagen gel. Additionally, Collagen tubes filled with collagen gel have promoted more rapid nerve sprouting and better morphology than saline-filled collagen tubes [30]. However, in some cases, collagen gels have hindered regeneration [31]. This negative effect, which presumably results from gel remnants blocking diffusion and axonal elongation, might be overcome by reducing the concentration of the collagen gel [32].

Hyaluronic acid, an ECM component, is associated with decreased scarring and improved fibrin matrix formation. It is hypothesized that during the fibrin matrix phase of regeneration hyaluronic acid organizes the ECM into a hydrated open lattice, thereby facilitating migration of the regenerated axons [33]. Hyaluronan-based tubular conduits, which are used for peripheral nerve regeneration, resulted in more myelinated axons and higher nerve conduction velocities than silicone tubes filled with saline with little cytotoxicity upon degradation [34,35]. Other gels used in vivo to promote nerve regeneration include Matrigel, alginate gels, fibrin gels, and heparin sulfate gels (Tables 69.1 and 69.2) [36–38].

Extracellular Matrix Molecules for Nerve Regeneration

Insoluble ECM molecules such as laminin, fibronectin, and forms of collagen promote axonal extension, and thus are excellent candidates for incorporation into the lumen of NGCs. Agarose gels cross-linked with laminin showed enhanced neurite extension from chick dorsal root ganglia (DRG) in vitro [29]. Agarose gels cross-linked with laminin and soluble nerve growth factor (NGF) showed nerve regeneration comparable to autografts over a 10-mm gap in rats [39]. However, axonal extension in the laminin gels depends on concentrations of laminin, and high

TABLE 69.1 Classification of Nerve Grafts: Examples and References

1. Isotropic grafts: have uniform distribution of one or more of the four components	
A: Scaffolds	
Natural materials	Veins [13,14], muscle fibers [20]
Synthetic materials	PLA [20a], PLLA, PGA, PAN/PVC [25]
Gels	Agarose [39], alginate [38]
B: Neurotrophic factors	NGF [43,44], BDNF [44a], IGF [44b], FGF [47]
C: Extracellular matrix proteins	Laminin [39], fibronectin [40], collagen [17]
D: Support cells	SCs [56], fibroblast [57], stem cells [16,54]
2. Anisotropic grafts: have directional distribution of one or more of the four components	
A: Scaffolds	
Aligned filaments	Collagen [38,75], PLLA [72]
Magnetically aligned gels	Fibrin [12], collagen [36,76]
B: Neurotrophic factors	NGF [79,80], BDNF [79]
C: Extracellular matrix proteins	Laminin [82,83]
D: Support cells	SCs [88,89]
3. Autologous nerve grafts: have all four components	[47] and [91]
4. Nerve allografts: acellular grafts	[93] and [92]

BDNF, brain-derived neurotrophic factor; *FGF*, fibroblast growth factor; *IGF*, insulin-like growth factor; *NGF*, nerve growth factor; *PAN*, polyacrylonitrile; *PGA*, polyglycolic acid; *PLA*, polylactic acid; *PLLA*, poly(L-lactide); *PVC*, polyvinylchloride; *SC*, Schwann cell.

TABLE 69.2 Classification of Nerve Guidance Conduits or Channels Based on Porosity and Degradability

Porosity	Degradability	Example and References
Impermeable	Nondegradable	Silicone [90]
Semipermeable	Nondegradable	PS [39], PAN/PVC [24,25]
Resorbable	Degradable	PLA [20a]

PAN, polyacrylonitrile; *PLA*, polylactic acid; *PS*, polysulfone; *PVC*, polyvinylchloride.

concentrations of laminin hinder regeneration [32]. Matrigel (a gel containing collagen type IV, laminin, and glycosaminoglycans) supports some degree of regeneration over a long nerve gap in rats when introduced into the lumen of NGCs [37]. Similarly, a gel mixture containing laminin, collagen, and fibronectin significantly improved nerve regeneration compared with saline-filled silicone channels [40]. Another approach to incorporating ECM signals into nerve conduits is the use of the RGD peptide, which is an amino acid sequence found in multiple ECM proteins [41]. Various research groups found that attachment of Arg-Gly-Asp (RGD) to collagen or poly([lactic acid]-*co*-[(glycolic acid)-alt-(L-lysine)]) conduits improved peripheral nerve regeneration [41,42]. Therefore, peptides and proteins that facilitate cell adhesion and migration within the scaffolds have an important role in cell−scaffold interactions and could be used to enhance nerve regeneration.

Neurotrophic Factors and Cytokine Delivery for Nerve Regeneration

Neurotrophic factors are produced in the target organs and by SCs in response to injury. These neurotrophic factors maintain the target organ−nerve synapse. A nerve injury usually results in disruption of communication between the target organs and the neuronal cell body and leads to Wallerian degeneration (the breakdown of myelin sheath and axons). The cytokines released during Wallerian degeneration activate SCs that produce neurotrophins such as NGF

and brain-derived neurotrophic factor (BDNF). Studies have shown that NGF production in target organs of sensory and sympathetic nerves in the PNS promote the survival of sensory ganglia and nerves, including spinal sensory nerves and sciatic nerves [43,44]. Also, it has been shown that BDNF expression is upregulated in injured peripheral nerves that promote the survival and outgrowth of sensory and sympathetic nerves as well as motor nerves [45]. Many other trophic factors, including insulin-like growth factor, fibroblast growth factor, and ciliary neurotrophic factor (CNTF), have been shown to be involved in the promotion of nerve regeneration [46,47].

Neurotrophic factors are likely an important part of future clinical therapies for peripheral nerve injuries and diseases. In diseases in which the functions of SCs are severely suppressed (multiple sclerosis, for example) or when acellular grafts (containing no viable SCs) are used, application of neurotrophic factors could be highly effective in facilitating nerve regeneration. Various studies have developed scaffolds for the sustained delivery of neurotrophic factors to enhance nerve regeneration. Hubble and Sakiyama-Elbert developed a fibrin matrix that immobilizes heparin molecules by electrostatic interactions, which in turn immobilizes heparin-binding growth factors. When implanted in vivo, the fibrin matrix releases the bound growth factor as a result of fibrin degradation [48]. This system was used to deliver NGF for peripheral nerve regeneration in vivo and basic fibroblast growth factor (bFGF) for neurite extension from chick DRG in vitro [45,48]. Fibrin−heparin−NGF matrix was observed to promote nerve regeneration comparable to syngeneic nerve grafts over a 13-mm nerve gap in rats. Fibrin matrix that released bFGF in vitro enhanced neurite extension from DRG by 100% compared with unmodified fibrin matrix. The use of PLGA microparticles for drug delivery has also facilitated the sustained delivery of neurotrophic factors such as glial cell line−derived neurotrophic factor (GDNF) and other cytokines such as erythropoietin (EPO) [49,50]. In a rat common peroneal nerve defect, sustained delivery of GDNF for 2 and 4 weeks using PLGA microparticles increased the axon counts and promoted larger fiber diameters comparable to autograft [49]. Another group found that delivering EPO using PLGA microparticles enhanced the functional recovery of a rat sciatic nerve defect by increasing the motor nerve conduction velocities [50]. Successful recovery using fibrin−heparin matrices and PLGA microparticles shows the importance of the temporal and spatial controlled delivery of neurotrophic factors and cytokines or nerve repair. Future research in this field may focus on developing biomaterials for the sequential delivery of neurotrophic factor and cytokines after peripheral nerve injury.

In addition to neurotrophic factors, other cytokines can be delivered to modulate aspects of the regenerative process after peripheral nerve injury. Scientists have focused on the effect of inflammatory and immune responses in the healing mechanisms of multiple tissues. In the context of peripheral nerve injury, scientists have found that macrophage polarization has an important role in nerve regeneration [51]. Macrophages can be classified across a spectrum in which the two ends represent inflammatory (M1) and antiinflammatory macrophages (M2). It is believed that macrophage phenotype, M1 or M2, could influence SC infiltration within the NGC and affect the healing process. The delivery of interleukin-4, a cytokine that switches macrophage population toward an M2 phenotype, from agarose scaffolds resulted in an increase in SC infiltration within the scaffolds and faster axonal growth in the rat sciatic nerve gap [51]. Another approach to creating an antiinflammatory environment within the injury site is the selective recruitment of M2 macrophages. M2 macrophages express high levels of fractalkine (CX3CR1); therefore, the delivery of CX3CL1 can be used for their selective recruitment. It has been shown that CX3CR1 delivery increased the infiltration of M2 macrophages and the number of regenerated axons at the distal end of the scaffold [52]. These results suggest that modulating inflammatory or immune responses may be a successful strategy to enhance NGC performance and promote successful nerve regeneration.

Seeding Neuronal Support Cells for Nerve Regeneration

In the PNS, SCs are support cells that wrap around the axons. SCs form a multilamellar sheath of myelin, a phospholipid-containing substance, around axons that serve as an insulator and increase nerve conduction velocity. In NGCs used for nerve regeneration, the formation of a fibrin cable, migration of SCs, and longitudinal arrangement of SCs (known as bands of Büngner) are necessary processes for axonal regeneration. For nerve gaps less than a critical length (10 mm), these processes occur spontaneously, leading to axonal regeneration. However, for nerve gaps greater than 10 mm, spontaneous nerve regeneration does not occur, because a fibrin cable and bands of Büngner fail to form [9]. SCs of uninjured nerves are quiescent. After nerve axotomy, the SCs become “reactive” and produce a number of neurotrophic factors, including NGF, BDNF, and CNTF [44,53]. They also synthesize and secrete ECM molecules such as laminin, which is known to modulate neurite outgrowth and express a variety of other cell adhesion molecules. All of these components have been suggested to have roles in supporting neuronal survival and axonal regeneration.

Using SCs in NGCs bypasses the fibrin cable formation step, accelerates the formation of bands of Büngner, and introduces a persistent source of neurotrophic factors, leading to more efficient nerve repair. This could decrease the time required by the axons to reconnect to the target organ and may increase the distance over which regeneration occurs. SCs isolated from the peripheral nerve of a patient and expanded in vitro could be used to treat the patient's nerve injuries. The addition of SCs has been shown to improve the performance of various scaffolds significantly, such as empty NGCs, collagen gels, venous nerve grafts, and muscle grafts, compared with control scaffolds without SC [18,54,55]. The ability of SC-seeded NGCs to promote regeneration was found to depend on the SCs' seeding density and immunocompatibility between donors and host [56]. For syngeneic SCs, it was observed that increasing the seeding density improves the nerve regeneration outcome. Heterologous SCs elicited a strong immune reaction, impeding nerve regeneration [56]. The performance of SC-seeded NGCs was further improved by designing longitudinally aligned channels in the tube to resemble acellular nerve grafts (see discussion on anisotropic scaffolds).

As an alternative to SCs, other cells could be used, as such or genetically modified, to produce desired levels of neurotrophic factors or to express specific ECM molecules. Fibroblasts, genetically modified to produce NGF, BDNF, neurotrophin-3 (NT-3), and bFGF, showed promising results in central nervous system (CNS) regeneration [57]. Olfactory ensheathing cells have been shown to promote the regeneration of cut nerves in the adult rat spinal cord [58]. Although these are examples of CNS regeneration, these genetically modified cells can be used for PNS regeneration as well. The addition of bone marrow stromal cells to NGCs has shown improved peripheral nerve regeneration over empty NGCs [16,17]. Similarly, pluripotent stem cells derived from hair follicles have shown improvements in rats [59]. However, the difficulties of isolating and culturing these cells from the patient before surgery could limit this approach for some surgical procedures.

Electroconductive Scaffolds for Nerve Regeneration

Electrical stimulation is another technique that has been used to promote nerve regeneration. Previous work showed that electrical stimulation of the soleus nerve of rabbits after a crush injury promoted twitch force, tetanic tension, and muscle action potential in soleus muscle, indicating enhanced nerve growth [60]. To elucidate how electrical stimulation accelerated nerve growth, various groups evaluated its effects on growth factors expression as well as other cellular responses. Electrical stimulation increased the expression of BDNF and tropomyosin receptor kinase B receptors on regenerating motor neurons [61]. Another group observed increased neurotrophin expression after electrical stimulation following nerve repair using a nerve allograft [62]. In addition to an increase in growth factor expression, it was found that stimulating motor neurons at 20 Hz for 1 h accelerated the sprouting of axons after nerve injury [61,63]. These results have motivated research groups to develop electroconductive scaffolds that create an electrical environment within large nerve gaps [64].

Electroconductive scaffolds can improve peripheral nerve injuries significantly. Polypyrrole (PPY) is an electroconductive polymer that has shown great potential as a nerve scaffold owing to its cell compatibility [65]. Various research groups have shown that scaffolds containing PPY increase the percentage of neurite-bearing cells and median neurite length [65,66]. Scaffolds made of PPY and poly(D,L-lactic acid) were able to repair a rat sciatic nerve injury in a way similar to autologous graft [65]. Another study found that conductive PPY—chitosan improved not only axonal regeneration but also remyelination. The researchers also found an improvement in the animals' motor and sensory functions [64]. The mechanism by which electrical stimulation enhances peripheral nerve regeneration still needs to be fully elucidated. However, future strategies could combine electrical stimulation with other scaffold components discussed throughout this chapter to accelerate successful nerve repair after a peripheral nerve injury.

ANISOTROPIC SCAFFOLDS FOR NERVE REGENERATION

The four essential elements of nerve scaffolds, neurotrophic factors, ECM molecules, and cells can be presented in an aligned fashion so as to orient the regenerating axons toward their distal targets. Several anisotropic scaffolds have been fabricated to affect neuronal behavior. Neuronal as well as glial cells respond to the underlying topographical cues in a particular manner. Distinct cellular processes, including migration, polarization, and gene expression, have been shown to be affected by the isotropy of tissue engineered scaffolds [67]. Although the exact mechanisms of how topographical cues affect cell behavior have yet to be elucidated, they have been hypothesized to affect protein attachment, orient cell cytoskeleton, and affect downstream gene regulation [68,69]. In this section, studies involving nerve grafts that provide directional guidance are discussed.

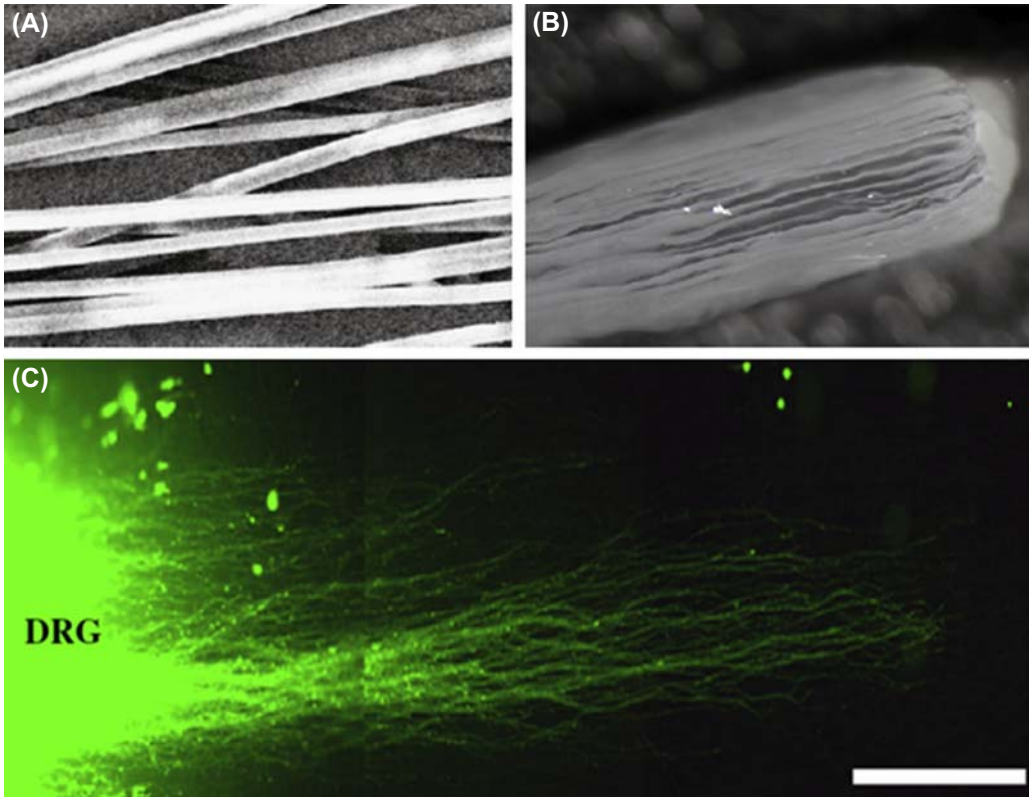

FIGURE 69.2 **Nanofilaments for contact guidance-mediated growth.** (A) Scanning electron microcopy image of PAN-MA nanofilaments with a diameter of 400–700 nm. (B) Three-dimensional nanofilament-based scaffold, along with agarose gel, embedded in a polysulfone nerve guidance channel can be used to direct neurite growth in vitro or axonal growth in vivo. (C) Chick dorsal root ganglia (DRG) extending neurites along horizontally oriented PAN-MA filaments, in vitro (scale bar = ¼ 1 mm. *PAN-MA*, poly(acrylonitrile-*co*-methylacrylate). *Images courtesy of Dr. Young-tae Kim, Georgia Institute of Technology.*

Aligned Anisotropic Scaffolds

We hypothesize that the superior performance of autologous nerve grafts is the result of its cellular components and its longitudinally aligned structure. The longitudinally aligned structure of the degenerating nerves in the autografts provides contact guidance and direction to the regenerating nerves. In an attempt to mimic autografts, longitudinally patterned or oriented gels and filaments to guide and accelerate the regenerating axons have been designed (Fig. 69.2). It has been shown that a poly(acrylonitrile-*co*-methylacrylate) nanofilament-based scaffold alone can facilitate regeneration across a 17-mm nerve gap in rats [70]. Many other combinations of materials have been used, such as collagen filaments embedded in collagen tubes, laminin-coated collagen fibers in collagen tubes, laminin-fibronectin double-coated collagen fibers in collagen tubes, poly(L-lactide) filaments in silicone tubes and PLA tubes, and PGA fibers in chitosan tubes [71–75]. All have been found to improve regeneration significantly compared with saline-filled tubes. In addition to synthetic filaments, magnetically aligned fibrin and collagen type I gels have been used to provide directional guidance to neurites in vitro and axons in vivo [36,76,77].

Neurotrophic Factors

Neurotrophic factors have been delivered mostly in an isotropic manner in vivo. However, in vitro studies suggested that gradients of neurotrophic factors can direct growth cones toward the source of neurotrophic factor [78]. Insoluble and soluble gradients of NGF, NT-3, and BDNF have been shown to direct the growth of neurites from PC12 cells toward increasing concentrations of neurotrophic factors [79,80]. In vivo, poly(ε-caprolactone-co-lactide) (PCLA) scaffolds with NGF gradients have been shown to improve nerve regeneration significantly better than isotropic NGF scaffolds [81]. Therefore, anisotropic scaffolds with gradients of neurotrophic factors, along with other components, might be an important tool for PNS regeneration.

Extracellular Matrix Molecules

Gels containing ECM molecules, such as laminin, collagen, fibronectin, and glycosaminoglycans, have been widely used to make isotropic scaffolds for nerve regeneration. ECM molecules promote axonal growth by the mechanism of differential adhesion, in which axons preferentially grow on substrates of ECM molecules owing to the presence of specific cell surface receptors. In vitro experiments with spatial patterns of whole ECM molecules or their peptide derivates have been used to direct the growth of neurites as well as enhance neurite extension [82,83]. Studies have demonstrated that gradients of ECM proteins could orient and enhance neurite outgrowth toward increasing concentrations of ECM molecules in vitro [82–85].

In vivo, ECM protein-coated fibers have been used to enhance nerve regeneration, in which the fibers provide contact guidance for regenerating axons and the ECM protein provides the adhesive substrate [71,73]. Also, anisotropic scaffolds created by gradients of laminin coupled to agarose along with sustained delivery of NGF from lipid microtubes matched autograft performance in terms of axonal diameter distribution after a peripheral nerve injury [86,87].

Cell-Seeded, Longitudinally Aligned Nerve Guidance Conduits and Channels

Neuronal-growth supporting cells can be incorporated with longitudinally aligned filaments and gels in NGCs to enhance nerve regeneration. Because support cells synthesize ECM proteins and neurotrophic factors, aligned cells often result in directionally aligned ECM. Biodegradable conduits of a copolymer of lactic and glycolic acids (PLGA) with longitudinally aligned channels have been used for nerve regeneration [88]. With the lumen coated with laminin and seeded with SCs, the channels showed regeneration comparable to nerve autografts over a 7-mm nerve gap in rats. PLA tubes with a micropatterned inner lumen and seeded with SCs, showed better nerve regeneration compared with unpatterned tubes with SCs [89]. However, disadvantages of cell-seeded NGCs include the need for prolonged isolation and cell culture to prepare cells for implantation, a high cell yield, and high cellular morbidity.

NATURAL NERVE GRAFTS

A common source of nerve grafts is the sural nerve, which is easy to obtain, has the appropriate diameter for most grafting needs, and is relatively indispensable. Other graft sources include the anterior branch of the medial antebrachial cutaneous nerve, the lateral femoral cutaneous nerve, and the superficial radial sensory nerve [90]. However, a motor nerve has a preference for a motor pathway (i.e., motor nerve graft) and shows inferior regeneration if a sensory nerve graft such as sural nerve is used. Similarly, a mixed nerve shows superior regeneration with either a mixed nerve or a motor nerve graft compared with a sensory nerve graft [91]. Therefore, clinical outcomes might be improved by using alternatives to sensory nerve grafts in reconstructing a mixed nerve. However, there are relatively few expendable motor or mixed nerves in the human body that could be used as graft materials. Therefore, a more feasible alternative would be to use nerve allografts or biosynthetic graft materials. Cadavers are an abundant source of graft materials and avoid the complications of harvesting autografts. However, cadaveric nerve allografts require maintenance and can be used only with immunosuppressive therapy. Withdrawal of the immunosuppressant leads to profound loss of axons in the allografts. The axonal loss is most profound in mixed nerve allografts compared with motor nerve allografts, followed by sensory nerve allografts [92].

Allografts, cold-preserved and/or freeze-thawed to prevent immune rejection by the host body, perform better than fresh allografts in terms of axon density, fiber diameter, and nerve conduction velocity [93]. Using natural materials (nerve grafts) for regeneration is ideal. However, it has been shown that if autografts or allografts are preserved for too long, their ability to support regeneration supporting is compromised [94]. Also, the pretreated allografts do not perform as well as autografts [93].

Although nerve autografts are used as the reference standard, the lack of functional recovery even with autografts remains an important clinical problem. Techniques employed to improve the performance of the nerve autografts include treatments to either remove the inhibitory molecules, such as chondroitin sulfate proteoglycans (CSPGs), or provide factors for axonal growth, such as NT-4/5 or BDNF. CSPG molecules have a core protein structure with glycosaminoglycan (GAG) side chains composed of chondroitin sulfate. Because of their large size and negative charge, the GAGs of CSPGs are thought to hinder neurite access to growth-promoting matrix molecules and repel the axons, inhibiting their growth [95]. It has been shown that CSPGs are upregulated almost sevenfold in the distal segment of peripheral nerve after transection nerve injury [96]. The upregulated CSPGs contribute significantly to

the inhibition of neurite sprouting and, consequently, growth into the distal nerve segment. Treatment of the injury site with chondroitinase ABC, which digests away the inhibitory CSPGs, increased the neurite ingrowth into the distal nerve segment several-fold compared with controls with no chondroitinase ABC treatment [97]. Treatment with chondroitinase ABC, however, did not improve neurite ingrowth in a crush injury model, which suggests that CSPGs are not upregulated in a crush injury.

Syngenic nerve grafts treated with chondroitinase ABC, heparinase I, heparinase III, or keratanase enzymes have shown significantly improved axonal ingrowth from the proximal nerve end into the nerve graft compared with untreated controls [98]. Autografts treated with a combination of all four enzymes showed the most significant neurite growth into the graft. However, combination treatment was not significantly different from the arithmetic sum of the individual treatments. This suggests that molecules such as heparan sulfate proteoglycan and keratan sulfate proteoglycan also contribute to the inhibition of neurite growth apart from CSPGs, and the pathways or mechanisms of inhibition for each of these molecules might be independent of each other. In another study, the exogenous supply of BDNF and NT-4/5 increased the number of axons regenerating into the nerve graft compared with untreated nerve grafts [99]. These techniques, used in clinical applications, could lead to better results with autografts or allografts.

Acellular grafts have been developed to address the problem of host response to allografts as well as the shortage of autografts. Cadaveric nerves are decellularized while keeping the ECM structure intact in order to promote nerve growth. It has been show that acellular cadaveric nerves from rats are able to bridge a 10-mm nerve injury [100]. In the same study, acellular grafts treated with NGF and vascular endothelial growth factor (VGEF) showed increased axonal diameter and neurovascularization at 1 month. Other studies have also shown that incorporating VGEF and NGF in acellular grafts promoted SC migration, increased the number of axons, and improved neovascularization compared with control [101]. In longer gaps (40 mm in Rhesus monkeys), acellular allogenic grafts seeded with autologous SCs enhanced nerve density and fiber counts [102]. Functionality of acellular grafts depends on the structural integrity of the graft after it has been processed for the removal of cells and immunogenic components. For this reason, several laboratories have developed chemical processing techniques that focus on preserving the integrity of the ECM structure. Studies with optimized acellular grafts created by Schmidt's group suggested that axon density after 84 days depended on graft structure and content [103]. In another study, processed acellular grafts held their laminin structure and promoted better functional results compared with nerve conduits in bridging nerve gaps [104].

ANIMAL MODELS

Traditionally, nerve regeneration studies involved the use of various animal models such as mice, rats, swine, canines, sheep, and nonhuman primates. Rat or mouse models are used initially to determine the efficacy of various treatments. If the results are encouraging, they are followed by experiments with larger animal models. For PNS regeneration studies, the most commonly studied nerve models are the sciatic nerve and its branches, the tibial and peroneal nerves. Other models include the cavernosal and facial nerves. The most common nerve injury model is the single-anastomosis model, in which the injury and repair are performed on one sciatic nerve and the contralateral sciatic nerve is used as a control. This model is useful when the nerve gap is not more than 20 mm. The second version is the cross (double) anastomosis model, in which both contralateral sciatic nerves are transected; the proximal end of the right sciatic nerve is then sutured to one end of an implanted tube and the distal end of the left sciatic nerve is inserted into the other end of the tube [105]. This model allows the study of gaps in excess of 25 mm. Although convenient, the rodent models have a serious drawback in that they present only short nerve gaps for regeneration studies. For a regeneration technique to be applied successfully in clinical trials, the nerve gap model has to be more than 40 mm long. To create a long nerve gap model, rabbits, cats, dogs, sheep, and nonhuman primates have been used [20,38,71,106,107]. The large-animal models are an important intermediary step before clinical application of experimental therapeutic approaches.

CONCLUSION

Despite significant advances in research into the development of synthetic NGCs, nerve autografts are still considered the first-choice strategy for nerve repair, especially in the case of long nerve gaps. However, performance of autografts themselves has been unsatisfactory. Use of autografts usually results in the good recovery of sensory functions but poor return of motor functions. Hence, there will be continued interest in ideas to enhance the

performance of autografts further by various treatments such as chondroitinase ABC, NT-4/5, and BDNF. However, the shortage of autografts and allografts is a major hindrance to their use. This shortage can be overcome by developing synthetic alternatives that can be tailored to enhance nerve growth. Modulating the spatiotemporal distribution of the four components of grafts can potentially improve the potential therapeutic outcomes of synthetic grafts. Ongoing rapid advances in cell biology, cell culture techniques, genetic engineering, and biomaterials research are likely to provide new tools to improve regeneration using synthetic grafts, and the day may be near when an engineered construct performs as well as autografts [1].

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CHAPTER

70

Regenerative Medicine for the Female Reproductive System

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INTRODUCTION

Normal embryonic development of the female reproductive system results from the presence of two X chromosomes, the absence of SRY gene expression (sex-determining region on the Y chromosome), and a complex network of gene interactions that determine the differentiation of the bipotential genital ridge into ovaries by 6–8 weeks of gestation [1,2]. In the genital ridge, primordial germ cells proliferate and enter meiosis to become oocytes [3]. Subsequently, internal genital development follows the female pathway in which a pair of Müllerian ducts (alternatively known as paramesonephric ducts) derived from invagination of the coelomic epithelium within the intermediate mesoderm is retained. The Müllerian ducts grow caudomedially to reach the urogenital sinus around the 10th week and its fusion and differentiation forms the fallopian tubes, uterus, cervix, and upper two-thirds of the vagina in the midline pelvis. At birth, the development of reproductive structures proceeds and complete maturation is achieved only at puberty when a cascade of hormone-regulated events results in adult physical characteristic and the capacity to reproduce.

Environmental or genetic factors may compromise organogenesis, resulting in a range of anatomical Müllerian duct malformations classified according to the developmental stage affected (duct formation, fusion, and septal reabsorption), with an estimated prevalence of 0.1–5% of the general female population [4–7]. Severe genital malformation such as Mayer–Rokitansky–Küster–Hauser syndrome (MRKHS) (abnormal or absent uterus, cervix, and upper part of the vagina) occurs in approximately 1 of 4000 newborn girls and can be associated with upper urinary tract and skeletal disorders [8–10].

Congenital and acquired diseases may adversely affect a woman's reproductive potential, leading to social and psychological problems [11–13]. Despite advancements in reproductive medicine and reconstructive surgery over the past decades, there is an increased demand for therapies that have the potential to restore function and/or compensate for the anatomical absence of female genital tissues and organs. Regenerative medicine has emerged as an attractive alternative to overcome limitations of current therapeutic approaches by integrating various technologies that range from tissue engineering to gene editing.

This chapter highlights progress in regenerative medicine and tissue engineering applications in the female reproductive system.

PRINCIPLES OF TISSUE ENGINEERING

Tissue engineering combines cell biology, materials science, biomedical engineering, and an appropriate environment to develop implantable biological substitutes that can restore and maintain normal organ function [14,15]. The development of a bioengineered tissue is a multistep process. As illustrated in Fig. 70.1, the first step is finding a viable immunocompatible cell source for the tissue of interest, preferably autologous, that can be isolated and expanded while maintaining the desired phenotype. When primary or progenitor autologous human cells cannot

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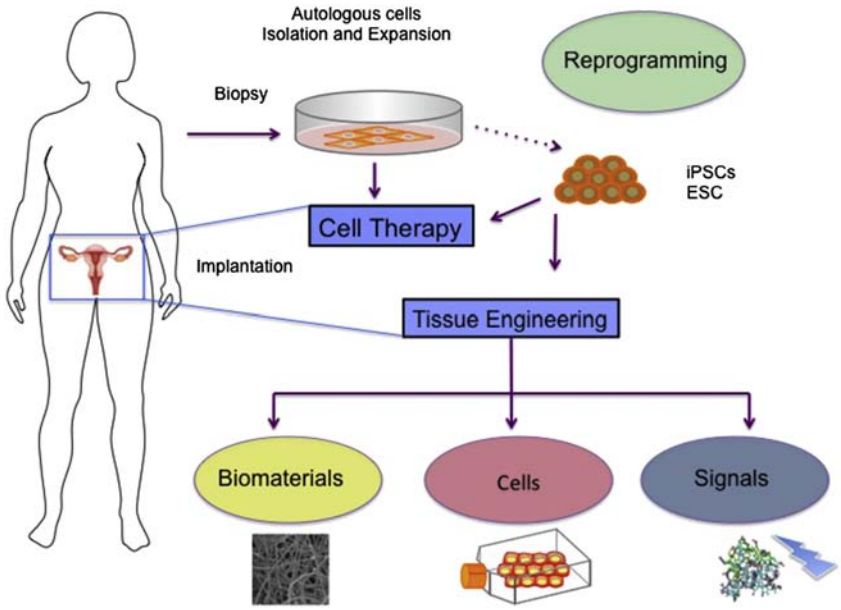


FIGURE 70.1 Schematic representation of tissue engineering and cell therapy approaches for the female reproductive system. *ESC*, embryonic stem cells; *iPSCs*, induced pluripotent stem cells.

be obtained from a particular organ, pluripotent human stem cells may be an alternative source given their potential to differentiate into a specialized cells type. Alternatively, multiples studies have shown that adult stem cells derived from the bone marrow, amniotic fluid, cord blood, or placenta have a great potential for regenerative therapies [16]. The next step in the process involves identifying a biocompatible material (scaffold) that supports cell growth in vitro and facilitates the complex process of tissue regeneration in vivo, withstanding the physiological and mechanical properties demanded by the organ [17]. In select circumstances, biomaterials alone or loaded with growth factors may be sufficient to replace native tissues. The constructs (scaffold plus cells) are usually incubated and/or preconditioned in vitro using a bioreactor system or directly implanted into the patient for in vivo tissue maturation.

THE VAGINA

The vagina is a distensible organ that extends from the vestibule of the vulva to the uterine cervix, which allows for sexual intercourse and serves as a channel for menstrual bleeding and childbirth. The vaginal canal is lined by a mucosa of nonkeratinized stratified squamous epithelium that has an important role as a barrier against harmful pathogens and harbors a dynamic microbiota that is shaped by ovarian hormone changes throughout a woman's lifetime [18]. The muscular layers consist of highly vascularized fibromuscular tissues and a network of nerves that are associated with pleasurable sensation during sexual activity [19].

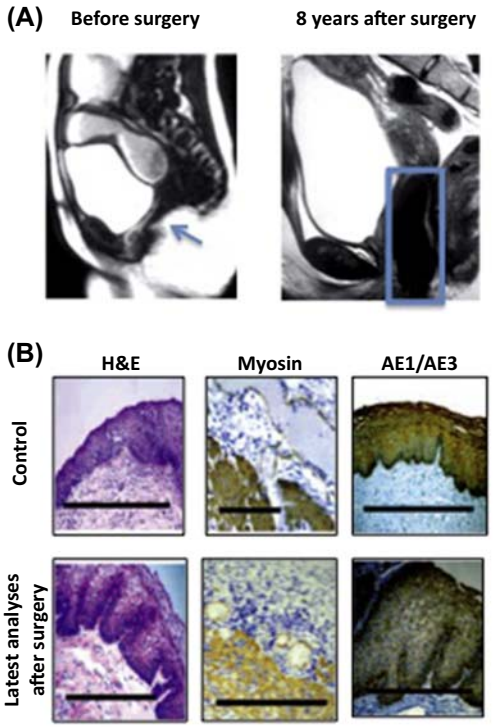
Acquired vaginal pathologies or congenital anomalies, including MRKHS, cloacal exstrophy, and complete androgen insensitivity syndrome, can adversely affect the vagina's anatomy, resulting in devastating psychosexual issues [20]. Nonsurgical vaginal dilation has been recommended as a first-line therapy but it is time-consuming, distressing, and unfavorable for severe conditions [21]. Another option is vaginal surgical reconstruction, which involves the creation of a canal (neovagina) between the bladder and rectum. A variety of vaginoplasty techniques have been described and a wide range of materials to line the surgically created cavity have been proposed including skin grafts, allogeneic tissue (amniotic membrane and epidermal sheets), peritoneum, artificial grafts, autologous buccal mucosa, and autologous in vitro cultured vaginal tissue [22].

Although no consensus has been established regarding what material should be used for reconstructive surgery, the application of nonvaginal tissue has been associated with complications such as graft rejection, chronic stenosis, and poor mechanical strength. The lack of materials that adequately mimic the vaginal tissue structure has led researchers to apply tissue engineering techniques to fabricate autologous vaginal tissue grafts [23].

Engineering of Functional Vaginal Tissue

Preclinical studies demonstrated the feasibility of engineering functional vaginal tissue using preconfigured scaffolds seeded with autologous vaginal epithelial and smooth muscle cells in a rabbit model [24,25]. We applied a similar strategy on a pilot cohort study to assess the use of autologous cell-engineered vaginal constructs in four patients aged 13–18 years with MRKHS [26]. Primary epithelial and muscle cells were isolated from 1 × 1-cm vulvar biopsies from each patient and expanded separately. Vaginal-shaped scaffolds made of decellularized porcine smallintestinal submucosa (SIS), a matrix rich in collagen and growth factors, were seeded with autologous cells at a density of 1×10^7 cells/cm^2 (for each cell type) and incubated for a week before surgery. The constructs initially measuring 7 × 10 cm were customized for each patient based on their pelvic cavity morphometric analyses. A vaginal reconstruction surgery was carried out for the autologous engineered vaginal construct implantation and a vaginal stent was put in place after surgery and kept for 2 weeks. Subjects were followed up for 8 years, and annual tissue biopsies, serial imaging, and clinical examinations confirmed vaginal tissue-like structures and long-term vaginal cavity patency (Fig. 70.2). In addition, all patients were sexually active and had functional variables for arousal, lubrication, orgasm, satisfaction, and painless intercourse within the normal range.

In another study, Panici et al. [27] reported the creation of an autologous in vitro cultured vaginal tissue for vaginoplasty in a 28-year-old woman with MRKHS. A 1-cm^2, full-thickness mucosal biopsy from the vaginal vestibule was taken and cells were expanded onto collagen IV–coated plates for 2 weeks. When autologous in vitro cultured cells reached 314 cm^2, they were mounted on hyaluronic acid–embedded gauze, maintaining the epithelial cell layer away from the gauze, and used to cover the surface of a surgically created vaginal canal. The gauze was kept in place by a vaginal mold. Thirty days after the procedure, the canal appeared normal in length and depth and a neovagina wall biopsy revealed epithelization resembling native vaginal tissue. The same group



(C)

	Score range	Sexual function index, individual patient score				
		Patient 1	Patient 2	Patient 3	Patient 4	All patients (average)
Desire	1.2–6.0	4.8	6	5.4	6	5.6
Arousal	0–6	5.1	5.7	5.8	5.6	5.6
Lubrication	0–6	4.8	5.7	5.7	5.7	5.5
Orgasm	0–6	5.6	6	5.6	6	5.8
Satisfaction	0.8–6	5.6	6	5.6	6	5.8
Pain (absesnce)	0–6	5.6	6	5.6	6	5.8
Full scale score	2–36	31.5	35.4	33.7	35.3	34.0
Average domain score	..	5.3	5.9	5.6	5.9	5.7

FIGURE 70.2 Tissue engineered autologous vaginal organs. (A) Preoperative and postoperative magnetic resonance images (*blue arrow* indicates the absence of a vaginal cavity and *blue box* shows an engineered vaginal organ). (B) Histological results of engineered vaginal segment biopsy samples at the latest yearly analyses after surgery (up to 8 years) showed a trilayered structure consisting of vaginal tissue with an epithelial cell-lined lumen surrounded by submucosa and muscle. Immunohistochemistry confirmed phenotypically normal smooth muscle and epithelia (control sample of normal vaginal tissue obtained from vaginal surgical discard specimen). (C) Female Sexual Function Index assessment showed variables within the normal range, as determined by the control subject values of the validated self-report instrument in all six separate domains measured such as desire, arousal, lubrication, orgasm, satisfaction, and painless intercourse. *H&E*, hematoxylin-eosin. AE1/AE3, pan-cytokeratin. Scale bar: 25μm. *Adapted from Raya-Rivera AM, Esquiliano D, Fierro-Pastrana R, Lopez-Bayghen E, Valencia P, Ordorica-Flores R, et al. Tissue-engineered autologous vaginal organs in patients: a pilot cohort study. Lancet July 26, 2014;384(9940):329–36. PubMed PMID: 24726478. Epub 2014/04/15. eng, with permission from Elsevier.*

reported a 12-month follow-up study in 23 women with MRKHS who underwent neovaginoplasty using the same approach [28]. At 3 months after surgery, neovaginal biopsies showed vaginal-like tissue, and at 12 months, all patients had satisfactory sexual quality of life.

Although the clinical experience to date is promising and provides a compelling demonstration of safety and feasibility, the technology for creating human functional autologous engineered vaginas is not yet ready for wide application and this procedure is still considered experimental.

THE UTERUS

The uterus is the largest organ of the female reproductive system. It has an inverted pear shape and is anatomically divided into the uterine body or fundus (upper part) connected on each side to the fallopian tubes and the lower segment where the cervix opens into the vaginal cavity. The average dimensions of the normally developed adult uterus are approximately 8 cm long, 5 cm wide (upper part), and 3 cm thick. Its histological elements include the inner layer called endometrium composed of luminal and glandular epithelium surrounded by stroma; the myometrium, consisting of oriented layers of smooth muscle cells; and the perimetrium, the serosa that envelops the organ.

The uterus has complex vascular, contractile, and immune systems that together, in response to cyclic ovarian hormonal cues, support sperm migration, embryo implantation, fetal nourishment throughout gestation, and intense contractions during childbirth [29]. The endometrium undergoes approximately 400 cycles of controlled cellular proliferation, differentiation, angiogenesis, breakdown, and shedding of the basal layer throughout a woman's reproductive life [30]. In addition, a dramatic process of uterine cell differentiation, hypertrophy, and hyperplasia occurs during pregnancy, increasing the uterus weight up to 10 times; and by 4–5 weeks postpartum it returns to nongravid weight [31]. Based on this dynamic tissue remodeling, evidence for the existence of adult progenitor/stem cells in the human uterus has been reported [32,33]. Abnormal endometrial progenitor/stem cell function and disruption of their surrounding niche may be associated with proliferative diseases such as endometriosis and endometrial cancer as well as inappropriate tissue remodeling, which can potentially affect embryo implantation and fetal development [34].

Despite the advancement in minimally invasive surgical procedures, hormonal therapies, and assisted reproductive technologies, congenital and acquired uterine disorders remain a contributor to infertility in approximately 2–8% of woman of reproductive age [12,35].

Gestational surrogacy is an available option to achieve genetic motherhood; however, it involves complex legal issues and is still not approved in many countries [36–38]. Human allogeneic uterus transplantation has been proposed, and it proves to be feasible and successful in terms of pregnancy. However, this approach raises concerns regarding donor shortage and adverse effects of immunosuppression [39,40].

The development of regenerative medicine strategies aims to restore, enhance, and maintain normal uterine function.

Uterine Tissue Regeneration

Initial experiments using synthetic polymer-based grafts to regenerate a section of a rat uterus showed that 6 weeks after implantation, nonabsorbable polytetrafluoroethylene (Teflon) elicits an intense local inflammatory response, leading to severe adhesions, obstruction, and poor tissue ingrowth; whereas endometrium-like tissue ingrowth was observed within biodegradable polyetherurethane/poly-L-lactide grafts, but they failed to provide luminal patency [41].

We investigated the possibility of engineering autologous uterine tissue using a biodegradable synthetic polymer seeded with primary cells [42,43]. Endometrium and myometrium cells were isolated from rabbit uterine horns and expanded in vitro separately. These cells were seeded in a stepwise fashion on the surface and lumen of preconfigured uterine-shape scaffolds formed from polyglycolic acid coated with poly-DL-lactide-*co*-glycolide (PLGA) in chloroform (5% w/v), which were then used to replace a sub-total excised horn (mesometrium was retained) in animals from which the cells derived. Six months after cell-seeded construct implantation, the neouterine tissue showed organized cellular and anatomical structures as well as expression of specific markers for epithelial, stroma, and smooth muscle cells. Breeding studies to assess in vivo functional responses of the engineered uterine tissue are being conducted.

In another study, Campbell et al. [44] used the peritoneal cavity as an in vivo bioreactor to produce an autologous graft. Tubular boiled blood clot templates of 2.5 × 0.5 cm were implanted into the peritoneal cavity of rats. After 2 weeks, the encapsulated myofibroblast-rich tissue resulting from foreign body response was harvested and used to replace approximately one-third of one uterine horn of the host animal. At 12 weeks, the grafted tissue developed organized morphology with luminal and glandular epithelium, and two distinct layers of smooth muscle bundles interspersed with collagen, resembling native tissue. The researchers reported that grafted uterine horns supported embryo implantation and fetal development to the late stages of gestation, with reproductive outcomes comparable to normal controls.

Allogeneic extracellular matrices have also been tested as biological scaffolds alone, cell-seeded scaffolds, or growth factor delivery vehicle. Ansari et al. [45] replaced a segment of the rabbit uterine horn with a specially prepared human umbilical vein. At 6 months after implantation, there was no evidence of host rejection; the luminal surface of the graft showed epithelial reorganization but there was minimal myometrial ingrowth. In another study, Taveau et al. [46] tested the ability of different lengths (0.5−2 cm) of porcine SIS membranes to regenerate a segment of a rabbit uterine horn. Approximately 8 weeks after surgery, histological analysis showed tissue ingrowth of all layers within SIS grafts but disorganized myometrium architecture. The authors examined tissue functionality by naturally mating animals 28 days after the surgical procedure. They reported that the ability to maintain luminal patency and support pregnancy was limited to the size of the grafts. Longer grafts did not provide enough mechanical strength and collapsed.

In another study, bovine skin-derived collagen membranes loaded with collagen-binding human basic fibroblast growth factor (bFGF) were used as scaffolds in a rat model [47]. A section of the uterine horn was removed (approximately 1.5 cm in length and up to two-thirds in circumference) and replaced by 1.5 × 0.5 cm collagen membranes. At 90 days after surgical implantation, the bFGF delivery system enhanced vascularization, improved tissue ingrowth, with luminal and glandular epithelium development and partially organized muscle bundles, and supported pregnancy.

Ding et al. [50] investigated the effects of collagen membranes loaded with donor rat bone marrow−derived mesenchymal stem cells (BM-MSCs) in a murine model of severe uterine injury. They used isolated $CD29^+$, $CD44^+$, $CD90^+$, $CD34^-$, and $CD45^-$ BM-MSCs that displayed multilineage mesodermal differentiation. Collagen scaffolds (1.5 × 0.5 cm) were seeded with labeled BM-MSC suspension (5×10^5 cells/cm^2 scaffold) and incubated for 1−3 h before being engrafted in a partially removed uterine horn. At 4 weeks after surgery, BM-MSCs were mostly located at the basal layer of the remodeled endometrium and the perigraft tissue expressed higher levels of bFGF, insulin-like growth factor-1, vascular endothelial growth factor (VEGF), and transforming growth factor-β1 compared with the collagen membrane-only group. At 90 days, neotissue showed increased blood vessel density and organized endometrium and myometrium structures, and supported embryo implantation and viable offspring with comparable size and weight to those from normal uterine tissue.

The application of decellularization techniques has been explored to create naturally-derived matrices for uterine tissue reconstruction. As an initial step, Santoso et al. [48] described different protocols to decellularize rat uterine tissue patches using sodium dodecyl sulfate (SDS), high hydrostatic pressure (HHP), or Triton-X at optimized conditions; and examined matrices' effects on in vivo uterine tissue regeneration. Histological results indicated efficiency upon cell removal and minimal changes in the overall matrix structure using SDS and HHP protocols whereas at the conditions used, Triton-X severely reduced collagen and elastin content within the matrix. At 30 days after the transplantation of 15 × 5-mm decellularized scaffolds into rat uteri, all uterine tissue layers were observed within the grafts, which were able to support pregnancy in both SDS and HHP groups.

Vascular perfusion technique using Triton X-100, dimethyl sulfoxide, and sodium deoxycholate has also been applied to create whole uterus scaffolds for tissue regeneration [49]. Miyazaki et al. [51] attempted to repopulate a whole rat decellularized uterine matrix injecting 5.1×10^7 neonatal uterine cells, 2.7×10^7 adult uterine cells, and 1.0×10^6 rat BM-MSCs in the uterine wall. After 3 days of incubation in a perfusion system, an endometrium-like tissue formation was observed, although cells were not evenly distributed within the matrix. When recellularized grafts measuring 1.5 × 0.5 cm were implanted in a partially excised rat uterine horn, uterine tissue ingrowth was noticed within the grafts and pregnancy outcomes were reported to be nearly comparable to animals with an intact uterus.

In another study, Hellström et al. [52] isolated and expanded donor primary heterogeneous uterine cells and mixed them with rat green fluorescent protein (GFP)-labeled MSCs to recellularize whole-rat uterus matrices. Patches of 2 × 0.5 cm were seeded through multiple injections of a cell mix (one primary uterine cell per 150

GFP-MSCs) and incubated for 72 h. In vitro analysis of recellularized scaffolds revealed limited cell distribution mainly restricted to the matrices' external surface. Recellularized patches of 5×10 mm were grafted in full-thickness excised uterine horns and animals were mated 6 weeks later. Uterine-like tissue ingrowth was described within the grafts, mainly from host cells (labeled GFP-MSCs could not be found in the transplanted construct), and remodeled uterine horns supported pregnancy and fetal development, although embryo implantation did not occur directly over the patches.

Autologous stem cell therapies targeting endometrial disorders such as severe intrauterine adhesions (Asherman syndrome) have been proposed and ultimately investigated in patients [53–55]. Santamaria et al. [55] conducted a pilot study in 16 women (aged 30–45 years) with refractory Asherman syndrome and endometrial atrophy. Patients received granulocyte–colony-stimulating factor infusion to mobilize BM-MSCs, and then CD 133^{+} cells (endothelial progenitor cell markers) were isolated from the peripheral blood. These freshly isolated cells were delivered (an average of 124.39 million cells) into the spiral arterioles via intrauterine arterial catheterization. After autologous BM-MSC injection, patients received hormonal therapy and endometrium functionality was assessed. The authors reported increased endometrial vascularization and menses intensity and duration in the first 3 months after treatment. Pregnancies were achieved spontaneously and through in vitro fertilization, with two live births of healthy babies at term after cell therapy.

Uterine Cervix Tissue Engineering

The cervix has important mechanical and protective roles during pregnancy and undergoes significant remodeling during parturition. An impaired uterine cervix can result in preterm birth, which is associated with significant perinatal complications. House et al. [56] demonstrated that tissue engineering strategies can be applied to create three-dimensional (3D) cervical-like tissue constructs by seeding human cervical cells on silk sponge scaffolds. Stromal cells were isolated from premenopausal women's cervical canal biopsies, seeded on a collagen-coated porous silk protein scaffold, and cultured under either static or dynamic conditions. After 8 weeks, cervical cells proliferated on a silk-based scaffold and synthesized an extracellular matrix with biochemical constituents and morphology that resembled native tissue. The dynamic culture condition was associated with significantly increased collagen deposition, glycosaminoglycan synthesis, and mechanical stiffness. These results suggest that human tissue engineered cervical-like constructs could be applied as an in vitro system to investigate cervical remodeling.

THE OVARIES

The ovaries lie on each side of the lateral pelvic wall and are connected to the uterus by the fallopian tubes [57]. The ovarian follicle is the functional unit of the ovary and is composed of an oocyte (germ cell) surrounded by layers of somatic follicular cells (granulosa and theca cells) and a basement membrane. At birth, the ovarian cortex contains a pool of primordial follicles formed during embryonic life that determines a finite reproductive life span [58]. Between puberty and menopause, hormonal signals recruit dormant primordial follicles, initiating a highly regulated process called folliculogenesis. Activated follicles increase in size over a period of weeks regulated by both endocrine and intraovarian mechanisms [59]. During folliculogenesis, granulosa cells proliferate and differentiate in several layers around the oocyte, theca cells differentiate into theca interna (androgen-secreting cells) and externa (connective tissue), and a fluid-filled cavity (antrum) is formed. At this stage, follicle growth and development rely on pituitary gonadotropins (follicle-stimulating hormone and luteinizing hormone). Gap junctions are formed between cells, facilitating communication between granulosa cells and the enclosed oocyte, which is essential for the maturation and release of an egg capable of fertilization.

At each reproductive cycle, ovarian follicles synthesize hormones (sex steroids and peptides) and a subset of dominant follicles that reach maturation and ovulation will undergo terminal differentiation and form the corpus luteum, which secretes progesterone, a hormone necessary to maintain pregnancy.

Systemically, ovarian hormones have a fundamental role in preparing the uterus for embryo implantation, mammary gland development, sexual function, skeletal growth, and bone homeostasis [60].

At the onset of menopause, a natural and permanent cessation of ovulation occurs, and the suppression of sex steroid hormones production may lead to vasomotor symptoms, sleep disturbances, urogenital problems, osteoporosis, and sexual dysfunction. Pathological loss of ovarian function may occur due to surgical resection, early

menopause, or ovarian failure in cancer-treated patients, impairing fertility and leading to an increased risk for cardiovascular diseases and osteoporosis.

In the attempt to restore ovarian physiological roles as a gonad and endocrine gland, several approaches applying regenerative medicine principles have been explored.

Tissue Engineered Ovarian Follicles

Human ovarian tissue banking has been proposed as an alternative to preserve fertility and future endocrine function in young women undergoing sterilizing chemotherapy or radiation [61]. Immature follicles have the greatest potential to be preserved and recovered from the freeze-thawing process [62]. However, after cryopreserved ovarian tissue transplantation, the onset of revascularization can take up to 5 days, and ischemia-reperfusion damage may induce graft follicle depletion [63]. Shikanov et al. [64] developed a delivery system combining hydrogels and angiogenic factors to enhance ovarian graft survival in mice. The ovarian cortical tissue was cryopreserved, thawed, and encapsulated in fibrin hydrogel modified with heparin-binding peptide (to provide a controlled release of heparin binding growth factors) loaded with VEGF. The encapsulated tissue was then transplanted into the ovarian bursal cavity. Results indicated that the delivery strategy enhanced graft vascularization, improved survival of primordial follicles, and enabled natural conception and live births.

When autologous transplantation of cryopreserved ovarian tissue carries the risk for reintroducing malignant cells into the patient, strategies for in vitro maturation of early-stage follicle have the potential to provide an additional option to preserve and restore fertility. Several in vitro culture methods for the follicle-enclosed oocyte maturation have been successfully investigated in mice and large animal species. However, the translation into human follicles has been challenged by their greater diametric sizes, creating the need for extended culture time [65,66]. Culture of early-stage human ovarian follicles in 2D systems has been associated with spatial disruption of cells, because the portion of the follicle that is in contact with the culture substrate attaches to the surface and granulosa cells migrate away from the oocyte, leading to flattened follicles [67–70]. To address 2D-related limitations, 3D culture systems have been developed to recapitulate the biological architecture of ovarian follicles and provide an environment that supports follicle growth and maturation [71,72]. Pangas et al. [73] pioneered the use of alginate as a hydrogel for in vitro culturing individual immature ovarian follicles in a mice model. Alginate is a naturally occurring polysaccharide, typically extracted from brown algae, and one of the most commonly used biomaterials for encapsulation owing to its biocompatibility, low toxicity, low cost, biomechanical properties, and ability to form gel in the presence of calcium ions [74,75]. Studies using alginate-based encapsulation techniques have demonstrated a preserved in vivo-like spherical follicle morphology and communication between the somatic and germ cells, creating a microenvironment that favors normal folliculogenesis and proper steroidogenesis [72,76–79]. Xiao et al. [80] described a two-step follicle culture method that supports human oocyte maturation. In the first step, secondary follicles were mechanically isolated from the patients' ovaries and encapsulated individually in 0.5% alginate hydrogels. The beads were cultured in 96-well plates; once the follicles reached the antral stage (400–500 μm), they were released and transferred to low-attachment plates and then cultured for up to 30–40 days. This two-step strategy aimed to recapitulate the dynamics of in vivo ovarian folliculogenesis, in which immature follicles gradually move from the rigid cortex zone to the less dense perimedullary region. The authors reported the development of fertilizable metaphase II oocytes using this in vitro maturation approach.

In another study, Krotz et al. [81] created a self-assembled artificial human ovary to mature early antral oocytes. Theca cells isolated from antral follicles of reproductive-aged women were seeded into agarose molds to form a honeycomb structure. Granulosa cell spheroids or cumulus–oophorus complexes (COC) were then placed in the honeycomb openings and the constructs were cocultured for up to 7 days. At 72 h, theca cells surrounded the COC, resulting in self-assembled microtissues that supported oocyte maturation.

Cell encapsulation techniques have also been investigated as a potential cell-based hormone therapy for menopausal symptoms. This delivery system aims to reduce unwanted side effects of hormone replacement therapy by providing an endogenous pulsatile hormone release regulated by the hypothalamus–pituitary–gonad axis. Ideally, the microcapsules should be biocompatible, provide a barrier between the allogeneic ovarian cells and the recipient to prevent immune rejection, and promote long-term cell survival. Sittadjody et al. [82] developed an engineered multilayer ovarian tissue that secretes ovarian hormones in vitro in response to follicle-stimulating hormone and luteinizing hormone (Fig. 70.3). Ovarian cells were isolated from immature rats and encapsulated in alginate hydrogel as a multilayer construct that recapitulates native follicular architecture. Using a microfluidic device, poly-L-ornithine–coated microcapsules containing granulosa cells were mixed with theca cells and

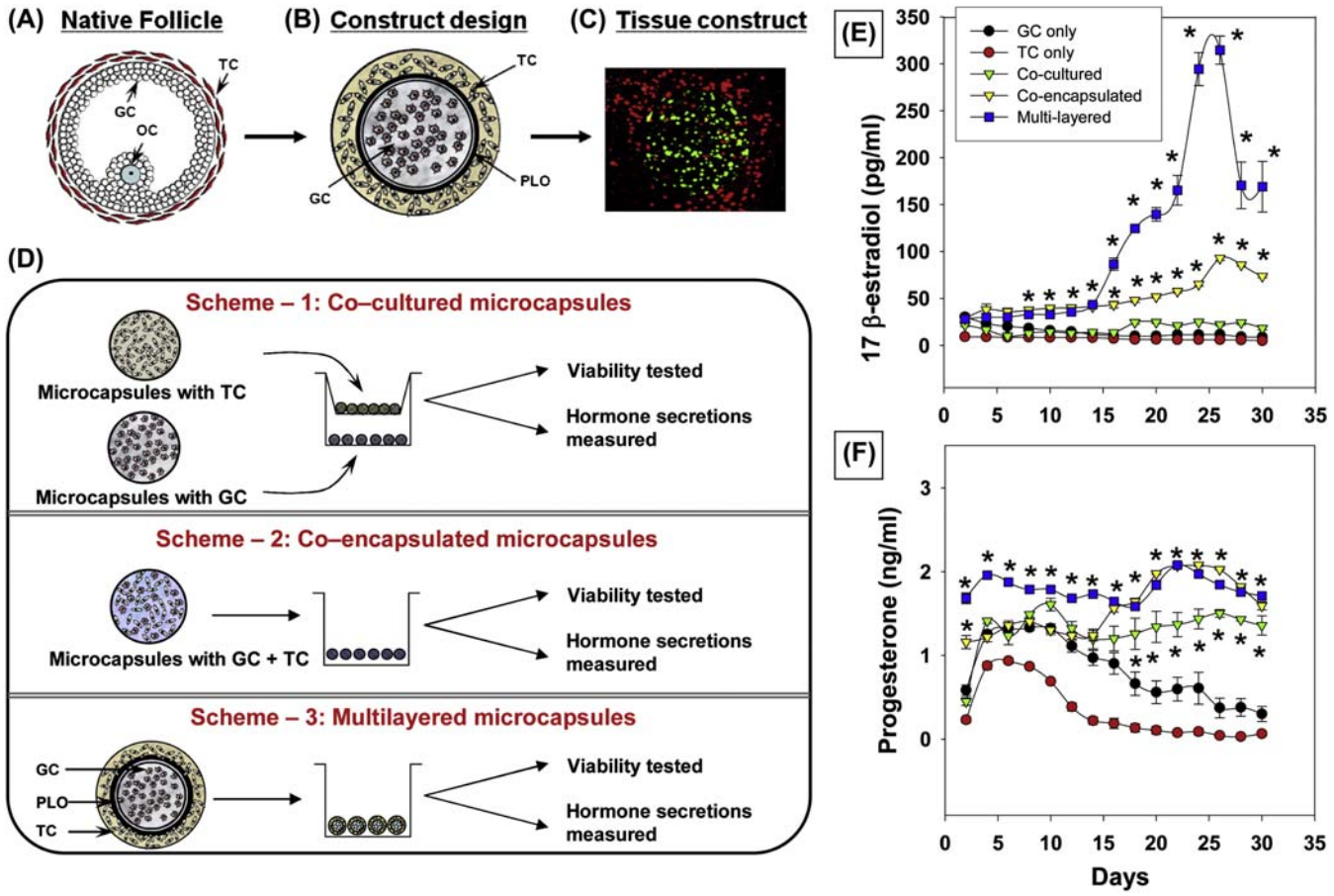


FIGURE 70.3 Encapsulation approach for reconstructing ovarian endocrine tissue: (A) Schematic diagram of a native ovarian follicle. (B) Multilayered alginate microcapsule design to mimic native follicular structure. (C) Three-dimensional (3D) confocal image of microcapsules demonstrating compartmentalization of different cells and distribution of CellTracker green-labeled cells (granulosa) in the inner layer and CellTracker orange-labeled cells (theca) in the outer layer. (D) Outline of the study demonstrating the cocultured/coencapsulated/multilayer microcapsules as the three approaches used. (E) Sustained E_2 and (F) P_4 secretion by granulosa and theca cells encapsulated using three different schemes. In contrast to 2D cultures, E_2 levels are maintained or increased with time and the presence of both GC and TC (through Schemes 1 and 2, or 3) led to increased levels of E_2 production compared with GC or TC alone. Multilayer cultures led to significantly increased levels of E_2 production. Each data point represents the mean ± standard error of the mean of six values (three wells per group assayed in duplicates). *Denotes statistical significance at $P < .05$ compared with granulosa and/or theca cells alone. The figures represent data from one of two separate experiments. *E_2*, 17β-estradiol; *GC*, granulosa cells; *OC*, oocyte; *PLO*, poly-L-ornithine; *P_4*, progesterone; *TC*, theca cells. *Adapted from Yalcinkaya et al. Yalcinkaya TM, Sittadjody S, Opara EC. Scientific principles of regenerative medicine and their application in the female reproductive system. Maturitas January 2014;77(1):12–9. PubMed PMID: 24210634, with permission from Elsevier.*

suspended in 1.5% alginate to be encapsulated. Multilayered constructs were cultured in the presence of gonadotropins and showed sustained secretion of sex steroids and peptide hormones over 30 days.

Microencapsulated ovarian cells have been reported to secret steroid hormones in vivo. Gou et al. [84] implanted approximately 2×10^6 microencapsulated allogeneic ovarian cells in the abdominal cavity of ovariectomized mice. Ninety days after transplantation, cells within the microcapsules survived and maintained the balance of bone absorption and formation by secreting estrogen continuously. Similarly, Liu et al. [85] co-microencapsulated granulosa and theca cells in alginate–chitosan–alginate and transplanted them into ovariectomized rats. Animals exhibited normal serum levels of estradiol and progesterone for 60 days and most of the retrieved microcapsules remained intact.

Regenerating Ovarian Tissue From Stem Cells

Studies in reproductive biology have challenged the belief that most female mammals are born with a finite number of oocytes that are incapable of postnatal renewal. The existence of germline stem cells (GSCs) in the juvenile

and adult mouse ovary was first postulated by Johnson et al. [86]. During an investigation of follicular atresia, a series of experiments revealed that based on the oocyte degeneration and clearance rates, proliferative germ cells are needed to support the animals' reproductive life span. The researchers identified a group of large ovoid cells in the surface epithelium of the ovary that immunostained positive for the specific germline marker mouse Vasa homolog (MVH) and incorporated 5-bromodeoxyuridine, thus confirming their proliferative potential. Furthermore, the MVH-positive cells expressed genes involved in the initiation of meiosis (synaptonemal complex protein 3). Finally, ovarian tissue from wild-type mice grafted into ovaries of mice with ubiquitous expression of GFP, contained GFP-positive germ cells surrounded by wild-type granulosa cells, indicating that proliferative germ cells sustain oocyte and follicle production in the postnatal mammalian ovary [86].

A study conducted by Zou et al. [87] made a major breakthrough claiming that postnatal mouse ovaries retain the capacity for oogenesis. Using a cell-sorting approach, GSCs, also referred as oogonial stem cells (OSCs), were isolated from the cortical layer of neonatal and adult mouse ovaries and cultured for long term. After being infected with GFP virus, these cells were transplanted into ovaries of infertile mice; and results indicated they underwent oogenesis and produced live GFP-positive offspring. In a subsequent study, White et al. [88] refined a fluorescence-activated cell-sorting protocol to isolate OSCs from mice ovaries and applied the same strategy into cryopreserved ovarian tissue from reproductive-aged women. Using an antibody against DEAD-box polypeptide 4 (the human ortholog gene of MVH), rare mitotically active cells were purified from human ovarian cortical tissue. These cells showed primitive germline profile, in vitro growth properties, and were able to generate oocytes in vitro and in vivo after xenotransplantation into immunodeficient female mice.

Nevertheless, the potential clinical relevance of the human equivalent of mouse OSC findings should be further explored because the incidence of OSCs in young adult mice ovaries is estimated to be extremely low, approximately 0.014% of all ovarian cells [88].

Extraovarian sources have been used to obtain germ-like cells including the bone marrow [89], fetal and newborn skin [90,91], mesenchymal cells [92], embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs) [93,94].

Hayashi's group achieved major progress in reproductive biology, reprogramming murine ESCs and iPSCs into fully functional oocytes in vitro [94]. Previously, Hayashi et al. [93] reported the ability to differentiate murine ESCs and iPSCs into primordial germ cell-like cells (PGCLCs) that can develop into functional oocytes when transplanted into immunocompromised female mice. After in vitro maturation and fertilization, these PGCLCs-derived oocytes contributed to fertile offspring [93]. In addition, a stepwise culture system was used to reconstitute in vitro the entire cycle of the mouse female germ line [94]. Starting with either ESCs or iPSCs, the expression of several genes was induced to create PGCLCs, which were then mixed with female gonadal somatic cells obtained from embryos to support germ cell in vitro development into mature oocytes. An in vitro fertilization protocol was used to test the oocytes' functionality, and the resulting embryos were transferred into surrogate females. The authors reported that only 3.5% of two-cell embryos derived from laboratory-grown oocytes resulted in viable offspring, which was much lower than that from in vivo−generated oocytes (61.7%), and the pups were healthy and grew into fertile adults. This culture technique may provide a platform to study molecular and cellular mechanisms of oogenesis of other mammalian species, including humans.

OTHER TISSUE ENGINEERING APPLICATIONS IN THE FEMALE REPRODUCTIVE SYSTEM

Pelvic Organ Prolapse

Pelvic organ prolapse (POP) is the abnormal descent of organs such as the uterus, bladder, urethra, intestine, and vagina that occurs when the pelvic floor supporting ligaments and muscles weakens. The estimated prevalence is approximately 30−40% of all women; pregnancy, aging, obesity, and genetic predisposition are the main risk factors. Commonly, these women experience symptoms of bladder, bowel, and sexual dysfunction that negatively affect their quality of life [95]. Surgical treatment options for POP involve reconstructive procedures with native tissue and/or the use of synthetic meshes to support the pelvic structural defect. However, mesh-related complications such as infection, mesh exposure, perforation, chronic inflammation, mesh shrinkage [96], and the lifetime risk for repeated surgeries [97] has led scientists to develop engineered grafts to enhance pelvic floor support and provide a more durable treatment. Hung et al. [98] engineered a fascia equivalent from isolated human vaginal fibroblasts. Full-thickness vaginal biopsies were taken from symptomatic POP patients and cells with high collagen I/III ratios were used for in vivo experiments, because they expressed greater proliferation potential. Vaginal fibroblasts were labeled with dialkylcarbocyanine fluorescent solution, suspended in collagen gel, and seeded

into biodegradable PLGA scaffolds. Fascia constructs were incubated in vitro for 5 days and implanted into immunodeficient mice subcutaneously. At 12 weeks after the surgical procedure, histological analysis of the grafts revealed the presence of labeled cells within a well-organized tissue formation, with an abundant collagen component, whereas poor tissue ingrowth was observed in the nonseeded collagen gel-coated PLGA grafts.

The use of cells derived from prolapsed vaginal tissues may not be ideal though; as they have shown altered in vitro characteristics including delayed fibroblast-mediated collagen contraction and lower production of collagen synthesizing enzymes compared with nonprolapsed tissue [99].

As an alternative autologous cell source, human endometrial mesenchymal stem cells (eMSCs) have been investigated in tissue engineering approaches for POP repair. Previously, human endometrial eMSCs were identified in a perivascular location in the human endometrium; characterized as multipotent, highly proliferative, and self-renewing stromal stem cells; and isolated using CD146, platelet-derived growth factor receptor-β, and sushi domain containing-2 (SUSD2) markers [33]. Using a xenogeneic model, Ulrich et al. [100] investigated the in vivo tissue responses and mechanical behavior of a novel nondegradable scaffold seeded with human eMSCs. In that study, SUSD2-enriched human eMSCs were labeled and seeded into a 10 × 25-mm polyamide mesh coated with 12% porcine gelatin at a density of 250,000 cells/mesh. According to the authors, polyamide mesh has biomechanical properties comparable to those of vaginal tissue. After 48 h of incubation, the constructs were implanted into the dorsum of immunodeficient rats and assessed at different time points up to 90 days. The cell-seeded mesh showed enhanced vascularization at day 7 after implantation; and at 90 days, results reveled a mild chronic inflammatory response, improved tissue organization, and minimal fibrosis around the seeded graft, which provided higher extensibility compared with nonseeded meshes. In addition, the organized collagen deposition was identified as being of rat origin, not human, which suggested that eMSCs exert a paracrine effect over the long term.

Future preclinical studies using clinically relevant models that represent a woman's pelvic tissue environment would be necessary to evaluate the efficacy and adverse effects of tissue engineered grafts for pelvic reconstructive surgery [101].

CONCLUSIONS AND FUTURE TRENDS

A successful pilot study of autologous engineered vaginal organs, with up to 8 years' follow-up, supports the potential of regenerative medicine technologies for treating female genital organs disorders. However, clinical trials involving a large number of patients are required to ensure that the most innovative therapies are as safe and effective as possible.

Advances have been made with engineering uterine tissue in preclinical models, but additional studies of biomaterials that produce adequately vascularized uterine tissue should be performed in systems that mimic the complex architecture and plasticity of the human uterus. In addition, important findings regarding the identification of human eMSCs with capabilities similar to those of BM-MSCs make the uterus an attractive source of stem cells that should be further explored for autologous cells therapy and tissue engineering applications.

Regenerative medicine principles have also been used to reconstitute the entire process of oogenesis in vitro and to engineer functional ovarian follicles, which represents a major achievement in reproductive biology. Nevertheless, challenges remain regarding the optimization of these in vitro culture systems for human gonadal cells.

Finally, the future of regenerative medicine applications for the human female reproductive system will rely on better understanding organogenesis and the complex physiological mechanisms of these organs to achieve the ultimate potential of these technologies.

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CHAPTER

71

Regenerative Medicine for the Male Reproductive System

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INTRODUCTION

The science of male reproductive function and dysfunction has experienced significant progress in recent years. In particular, clinical applications of molecular medicine to male infertility and hypogonadism [1], the "invention" of intracytoplasmic sperm injection (ICSI) as a treatment of male infertility [2], and the introduction of effective oral medication for erectile dysfunction (ED) [3,4] can be considered major breakthroughs. Infertility affects around 15% of couples who wish to have children, and half of these cases are associated with male-related factors [5]. ED is defined as a consistent or transient disorder; inadequate erection affects at least half of men aged 40–70 years, whereas chronic ED affects about 5% of men in their forties and up to 25% of men by age 65 years [6,7]. Hormonal and surgical approaches have been used to treat patients with reproductive system disorders. Regenerative medicine has become a promising concept in the development of new therapies for all fields of medicine, including reproduction [8]. The field of tissue engineering and regenerative medicine covers different areas of biomedical technology, including biomaterials, tissue engineering, and stem cell therapy. Three main strategies have been employed: (1) using extracellular matrices (ECM) without cells, allowing the natural ability of the body to generate new tissue, (2) using ECM seeded with cells for implantation, and (3) directly injecting cells with or without carriers such as hydrogels. ECMs used for tissue engineering may be either synthetic or derived from natural sources [9,10]. Different cell types may also be used, including (1) tissue-specific stem cells; (2) mesenchymal stem cells (MSCs); and (3) pluripotent stem cells such as embryonic stem cells, induced pluripotent stem cells, or amniotic and placental-derived stem cells. These cells can be either autologous or allogeneic. Autologous cells derived from a small biopsy are the preferred source because of immune compatibility. The biopsy is obtained from the patient and the cells isolated from this biopsy tissue are expanded in vitro. However, for some patients this is not an option because there is not enough functional tissue to obtain a biopsy. Therefore, other sources of cells, such as stem cells (either autologous or allogeneic), might be used for these patients [9,10]. The ultimate goal of regenerative medicine strategies to treat reproductive system disorders is to restore normal sexual function and preserve fertility.

TESTES

The hypothalamus, pituitary, and testes form an integrated system responsible for adequate secretion of male hormones and normal spermatogenesis. The general anatomy of the testis consists of the seminiferous tubules and, among them, the interstitial space [11]. The seminiferous tubules, which contain germ cells at different maturation levels, depend on age. The pool of germ cells is supported by spermatogonial stem cells (SSCs), which are covered by a layer of Sertoli cells embedded in lamina propria [12]. The lamina propria consists of the basal membrane covered by peritubular cells. Differentiated germ cells including spermatocytes and spermatids are located

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across the tubule toward its center. The main component of the interstitial space is the Leydig cell population, which produces testosterone; the interstitial space also contains macrophages, lymphocytes, loose connective tissue, and neurovascular bundles [13].

Spermatogonial Stem Cell Technology

Etiologies of nonobstructive azoospermia include endocrinopathy, testicular failure, genetic anomaly, and idiopathic factors. On average, every day in the United States, about five men become azoospermic owing to the adverse effect of cancer treatments [14]. In sexually mature boys and men with cancer, who are at risk for infertility, sperm cryopreservation is the simplest and most effective method to preserve fertility [15]. However, less than 25% of these patients apply for sperm banking [16]. Lack of knowledge about semen cryopreservation is a major reason for the low percentage of sperm storage [16]. However, because the production of sperm has not yet started in prepubescent boys [17], sperm banking is not an option for prepubertal cancer patients. On average, more than 14 boys aged less than 15 years are diagnosed with cancer every day and more than 80% of them will be cured [18]. Therefore, long-term infertility will be a critical issue for many of these childhood cancer survivors who have no option for sperm banking. SSCs are present in the testis of most mammalian species, including nonhuman primates and humans [19]. Spermatogonia are cells located in the basement membrane of seminiferous tubules in the testis (Fig. 71.1); a small subpopulation of them are SSCs that have a primary role in spermatogenesis [19]. Normally, SSCs are able to divide and differentiate into haploid germ cells (sperm) and maintain fertility in men during life [19].

In 1994, the feasibility of isolating and transplanting SSCs to restore fertility in mouse testes was established [20]. Since then, several groups have been successful in transplanting SSCs in other species including nonhuman primates [21]. This has given great hope for finding an effective alternative to preserving fertility in adult males and may also be a solution for restoring fertility in prepubescent boys after cancer treatment. In addition to cancer survivors, patients who may benefit from SSC implantation include but are not limited to those with cryptorchidism (at risk for infertility because of undescended testes at birth) and Klinefelter syndrome (47XXY; at risk for testis fibrosis) [19].

In 2002, Nagano and colleagues [22] isolated human SSCs from six infertile men and transplanted the cells to busulfan-treated nude mice. It was shown that human SSCs migrated into the base membrane of mouse seminiferous tubules of testis, but their numbers decreased significantly 1 month after transplantation. However, some SSCs remained in the mouse testis for up to 6 months with no evidence of differentiation [22]. A xenotransplantation study of prepubescent human SSCs showed results similar to those of the previous study on adult human SSCs [23]. The feasibility of SSC transplantation in a nonhuman primate model was demonstrated by Hermann and colleagues [21]. In that study, ejaculatory lentiviral positive sperm was found in the testes of more than 75% of adult (9 of 12) and 60% of matured prepubescent (two of five) recipient macaques monkeys for up to 63 weeks (average of 40.1 $\pm$ 4.9 weeks) after lentivirus-marked SSC transplantation. Autologous transplantation was twice as successful (70.5%; 12 of 17) than allotransplantation (33.3%; two of six) in terms of the presence of sperm in the ejaculate [21]. The ability of these sperm to fertilize eggs and develop preimplantation embryos was confirmed by performing ICSI. However, full pregnancies and live births were not observed in that study [21]. These results suggest that this

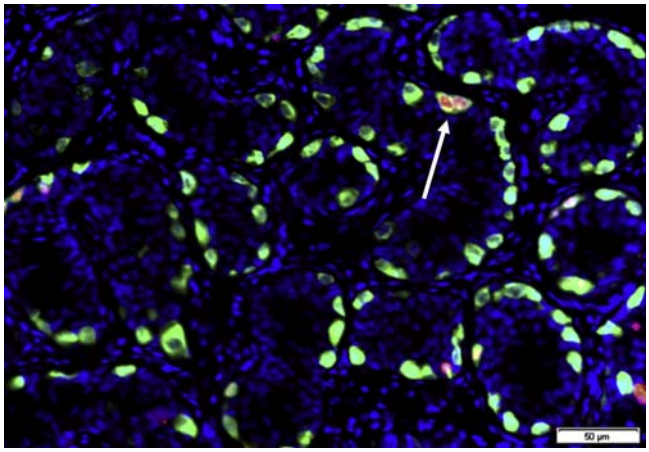

FIGURE 71.1 Spermatogonia cells (green fluorescent) in the base membrane of seminiferous tubules of a 5-year-old boy. The cells were stained by melanoma-associated antigen A4 antibody as a spermatogonia marker. Some of the spermatogonia cells have already started proliferating (*white arrow*; stained by Ki67 antibody). 4′,6-diamidino-2-phenylindole (*blue*) identified the nuclei of the cells. Scale bar = 50 µm.

technique could be translated to a clinical application in humans. In that study, an average of 88 ± 17.1 and 45.8 ± 14.5 million viable cells were used for transplantation to adult and juvenile testes, respectively [21]. It is well-known that increasing the number of cells corresponds linearly with the SSC transplantation success rate [24]. The number of SSCs in testis is low (0.03% of germ cells and 1.25% of spermatogonial cells in mice; unknown in humans) [25,26], and the size of testicular biopsy from premature boys undergoing cancer treatment is also small (around 100 mm^3; containing 30,000/mm^3 spermatogonia) [19,27]. Although human SSC autotransplantation has not yet been tried, it is expected that successful human SSC autotransplantation requires in vitro expansion of these cells before transplantation. This culture method had been developed in animal models [26,28–30]. In a study involving the isolation and culture of human testicular cells from six adult men who underwent orchiectomy as part of prostate cancer treatment, we were able to maintain and propagate human SSCs in vitro for longer than 20 weeks [31]. The number of SSC increased more than 18,000-fold over 64 days of in vitro culture. In a follow-up study using testicular tissue from two boys aged 6.5 and 8 years, who were diagnosed with Hodgkin lymphoma, isolated SSCs were able to propagate in vitro for at least 15.5–20 weeks [32]. Other groups have been successful in culturing human SSCs [33–36]; however, this culture system should be optimized under Good Manufacturing Practice regulations.

Before the first clinical trial of SSC autotransplantation in human, several safety issues must be addressed. First, especially in nonsolid cancer survivors such as hematopoietic malignancies, transplanted cells should be free of malignant cell contamination that may reintroduce the cancer to the recipient. In a study on culturing acute lymphoblastic leukemia (ALL) cells combined with testicular cells from three patients, ALL cells were undetectable beyond 26 days of culture even at an extremely high initial concentration (40% ALL cells) [37]. However, it will be beneficial to test more cell lines from different types of leukemia to ensure complete purging of malignant cells. Second, the genetic integrity of stored, isolated, and propagated SSCs is important because it can influence the next generation of offspring. It has been demonstrated that in vitro expanded mouse SSCs showed normal karyotype and stable androgenetic imprinting over 24 months [38]. Our work confirmed the genetic stability of in vitro propagated human SSCs; however, we found some changes in the epigenetic status of human SSCs during culture [39].

Although it is ideal to try for natural conception after SSC autotransplantation, in vitro spermatogenesis followed by ICSI is another option to preserve fertility in many patients. In vitro spermatogenesis models have moved forward from initially conserving anatomical structure of the testis in culture to complete meiosis from testicular mouse germ cells [40]. We developed a three-dimensional (3D) testis organoid system from adult human testicular cells (human testis organoid [HTO]) [47]. These HTOs consist of SSCs, Sertoli, Leydig, and peritubular cells (Fig. 71.2A). Each

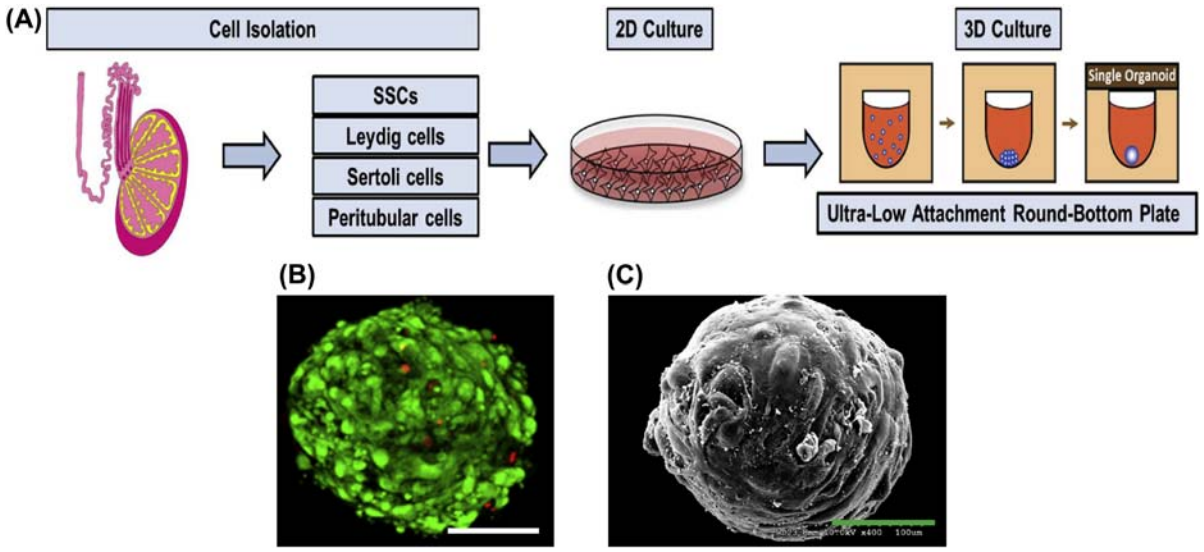

FIGURE 71.2 Human three-dimensional (3D) testis organoid system (HTO). (A) Testicular cells, including spermatogonial stem cells (SSCs), Leydig cells, Sertoli cells, and peritubular cells, are isolated and propagated in 2D culture and then seeded into ultralow attachment round-bottom plates for the 3D formation of organoids. The HTO formation takes 48–72 h. (B) Live/dead staining of organoid revealed maintenance of viability throughout the culture period. Green and red fluorescents represent alive and dead cells, respectively. (C) Ultrastructural analysis of multicellular human testicular organoids using scanning electron microscopy to show exterior morphology. Scale bars = 100 μm.

organoid is approximately 300 μM in diameter and remains viable for at least 3 weeks (Fig. 71.2B and C) in an enriched medium with testicular ECM. This organoid system mimics in vivo spermatogenesis by supporting the differentiation of SSC to postmeiotic germ cells, albeit at a low frequency of approximately 0.2% [41]. The feasibility of using this system should be tested on prepubertal human testicular cells, and then the fertilization potential of the differentiated haploid cells should be analyzed.

Androgen Replacement Therapy

Testosterone is the primary male sex hormone that has multiple roles in the body. It has important roles in developing male reproductive tissues such as testes and prostate as well as promoting secondary sexual characteristics such as increased muscle and bone mass and the growth of body hair [42]. Patients with testicular dysfunction and hypogonadal disorders may need androgen replacement therapy to restore and maintain physiological levels of serum testosterone and its metabolites. Therefore, testosterone therapy can increase muscle strength, stabilize bone density, improve osteoporosis, and restore secondary sexual characteristics, including libido and erectile function [43]. However, long-term exogenous testosterone therapy is not optimal and can cause multiple problems, including excessive erythropoiesis, bone density changes, and even infertility [44]. To address these problems, researchers are looking for an effective method to provide sufficient endogenous testosterone. In an animal model, isolated Leydig cells from rats that were cultured and stimulated with human chorionic gonadotropin (hCG) showed a high level of testosterone production [45]. In that study, engineered alginate-poly-L-lysine–encapsulated Leydig cell microspheres (with an average diameter of 0.7 $\pm$ 0.06 mm) were injected into castrated rats. Approximately 10% of a normal adult rat Leydig cell population was injected into each castrated animal. Serum testosterone levels increased and were maintained up to 40% of normal for a maximum of 43 days with no hCG stimulation [45]. Bilateral testicular agenesis or atrophy often requires placing testicular prostheses and androgen supplementation [46]. In this regard, the possibility of creating hormone-releasing testicular prostheses was investigated [47]. To form testicular prosthesis, isolated chondrocytes from bovine articular cartilage were seeded on testicular-shaped polymer scaffolds. Implanted engineered testis prostheses loaded with 100 μg testosterone enanthate were able to maintain physiologic levels of testosterone in vivo for at least 16 weeks in recipient mice [47]. For a better outcome, combinations of Leydig cell technology and engineered testicular prosthesis need to be explored further.

As mentioned, we developed a 3D testis organoid system from adult HTOs [41]. This HTO system consists of SSCs, Sertoli, Leydig, and peritubular cells. The Leydig cells in this multicellular structure could produce androgen with and without hCG stimulation [41] (Fig. 71.3).

Future work should focus on the feasibility of implanting adequate numbers of these HTOs to castrated animal models as a potential novel androgen replacement therapy.

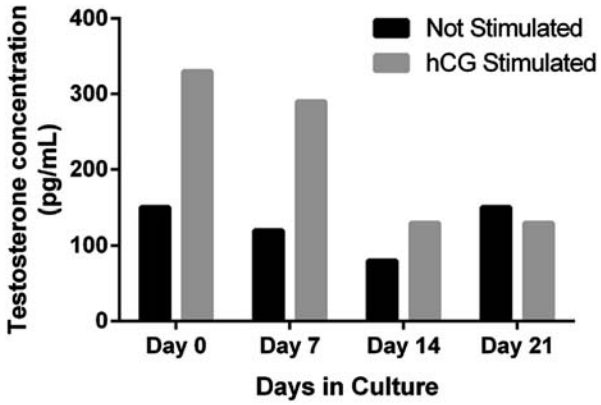

FIGURE 71.3 Androgen production by human three-dimensional testis organoid system. Testosterone concentration in organoid culture media was measured over 3 weeks. Organoids were treated with human chorionic gonadotropin (hCG) to stimulate testosterone production. Media from organoids treated with hCG were compared with control media from organoids with no hCG treatment. On days 0, 7, and 14, organoids produced more testosterone when stimulated with hCG. However, on day 21, organoids did not produce more testosterone when stimulated with hCG. The lack of increase in testosterone production on day 21 in response to hCG stimulation could have resulted from organoids becoming desensitized to hCG (at each time point, secreted hormones from a group of 16 organoids were pooled; n = 16).

EJACULATORY SYSTEM

Ejaculation is the process of transporting sperm through the urethra and expelling semen from the urethral meatus. The organs involved in the ejaculatory process are the epididymis, vas deferens, prostate, seminal vesicles, bladder neck, external urethral sphincter, and bulbourethral glands. In normal ejaculation, anatomic structures are precisely coordinated via neural centers to allow normal ejaculation to take place [48,49].

Engineering Vas Deferens

Congenital bilateral absence of the vas deferens (CBAVD) causes obstructive azoospermia, which is common in about 1% of infertile men [50]. Because CBAVD men have normal spermatogenesis, they are able to achieve biological fatherhood with the help of assisted reproduction techniques, i.e., surgical sperm extraction and in vitro fertilization (IVF) [51]. In addition, conception requires the female partner of men with CBAVD to undergo ovarian stimulation for an IVF/ICSI procedure. A solution to this problem would be to replace the vas deferens. A novel approach for the tissue engineering of vas deferens was tried in a rabbit model [52]. Polyethylene tubes (0.86 mm in diameter and 6 cm long) were implanted into the peritoneal cavities (as an in vivo bioreactor) of 16 rabbits [52]. After 2–3 weeks, the tubes were removed and the encapsulating myofibroblast-rich tissue that resulted from the foreign body response to the biomaterial was harvested for grafting as vas deferens in the rabbits [52]. At 2, 4, 6, and 8 months after implantation, morphological and immunohistochemical evaluations showed that the structure of engineered vas deferens was similar to that of native vas deferens tissue [52]. In addition, the presence of sperm in the ejaculate indicated normal function of the engineered vas deferens. However, more research on large-animal models is required before this approach can be used in humans.

Urethra Reconstruction

The male urethra is a narrow fibromuscular tube that conducts urine and semen from the bladder and ejaculatory ducts, respectively, to the exterior of the body. Many surgical procedures, such as grafting to replace damaged areas of the male urethra, may eventually fail. Various strategies have been proposed over the years to regenerate urethral tissue in several animal models, including woven meshes of synthetic polymers such as polyglycolic acid (PGA) without cells [53,54] and with cells [55], naturally derived collagen-based materials such as decellularized bladder submucosa [56], and acellular urethral submucosa [57]. The effectiveness of using collagen-based inert matrix was confirmed clinically in a series of four patients with a history of failed hypospadias reconstruction. Twenty-two months' follow-up showed successful repair of urethral defects with human bladder acellular collagen matrix in three of four patients [58]. In another study of 30 patients with recurrent stricture disease, human demineralized bone matrix obtained from cadaveric donors was processed and prepared for use as an off-the-shelf material [59]. Up to 36 months' follow-up showed that a healthy urethra bed (two or fewer prior urethral surgeries) was needed for successful urethral reconstruction using the acellular collagen-based grafts. In an advanced study with more than 10 years' follow-up [60], human clinical trials with a collagen-coated PGA tubularized scaffold seeded with autologous cells were conducted in a pilot series of five patients. The scaffolds were seeded with autologous cells derived from bladder biopsies taken from each patient. The cell-seeded scaffolds were then matured in an incubator and used to repair the urethral defects present owing to trauma. Long-term follow-up evaluation showed normal-range urinary flow rates and voiding cystourethrograms indicated that these patients maintained wide urethral calibers. Yearly biopsies showed that the grafts had a histological architecture consistent with normal urethral tissue (Fig. 71.4).

Spinal Ejaculation Generator

The incidence of anejaculation in the population is small, but when it is present, it represents a formidable challenge to the infertile couple and the treating physician [61]. Spinal cord injury (SCI) is the most common cause of anejaculation encountered in clinical practice [62]. The incidence of traumatic SCI is estimated at 16 individuals per million per year in Western Europe and 39 individuals per million per year in North America, with a prevalence of 300 and 853 individuals per million, respectively [63]. In the United States, mean age at occurrence of SCI is 37.1 years and 77.1% of SCI patients are male [64]. Ejaculation can be physiologically defined as the rhythmic, forceful expulsion of semen at the urethral meatus. Ejaculation is composed of two successive phases, emission and expulsion, each involving different pelvic-perineal anatomical structures [65]. Emission is controlled by autonomic

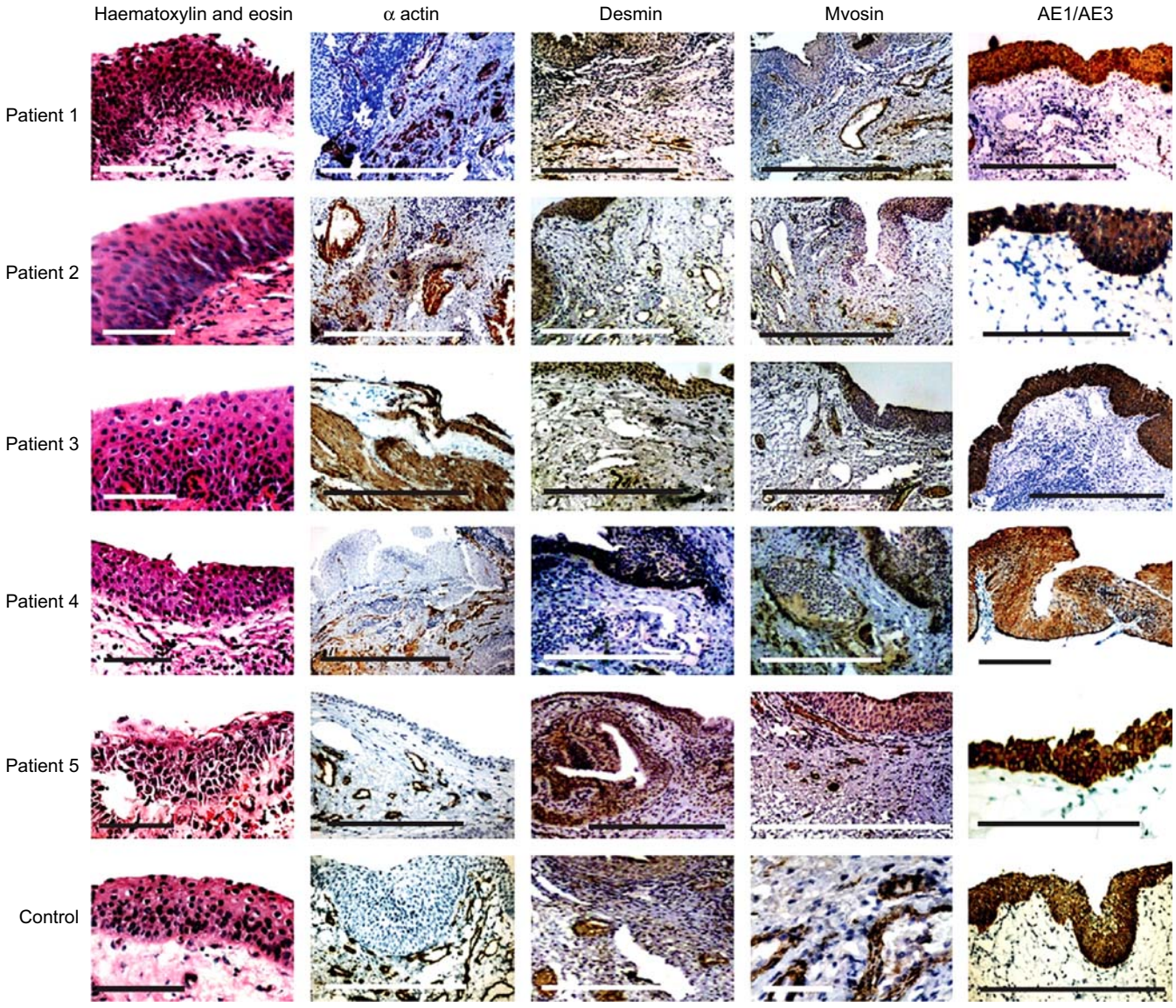

FIGURE 71.4 Histological and immunocytochemical analyses of implanted engineered urethra. Results of urethral biopsies of engineered segments from every patient at 1 year after surgery and from a control sample of healthy urethral tissue. *AE1/AE3,* Anti-pan Cytokeratin antibody. All scale bars = 25 μm.

(sympathetic and parasympathetic) spinal centers, and expulsion is controlled by somatic spinal centers. These centers act in synchrony for antegrade ejaculation to occur. In rats, such synchronization has been reported to be led by a group of lumbar spinothalamic neurons forming the spinal generator of ejaculation (SGE). The critical role of the SGE and its organization have been described in functional and neuroanatomical studies [65–69]. Penile vibratory stimulation (PVS) is a procedure that uses external vibration to stimulate the sensory nerves on the penis, with resultant reflex ejaculation (Fig. 71.5A). Electroejaculation is an option for men with SCI who have lower motor neuron lesions and for those who have failed PVS, but it is more invasive. This technique uses electricity delivered via a rectal probe to recruit the ejaculatory reflex. The Seager Electroejaculator (Dalzell Medical Systems, The Plains, VA) remains the only US Food and Drug Administration–approved device for this procedure (Fig. 71.5B).

Numerous studies have focused on therapeutic approaches of SCI in animal models. Significant progress on functional remyelination [70], direct injection of biological agents [71], formation of neuronal relays [72], biomaterial approaches to enhancing neurorestoration [73], and neural stem cells transplantation [74,75] has been made. These studies promise translation to more clinical trials on SCI and neuronal regeneration. Although the main aim of these studies is to correct motor and sensory deficits, it will be interesting to evaluate improvement in ejaculation function of these patients, as well.

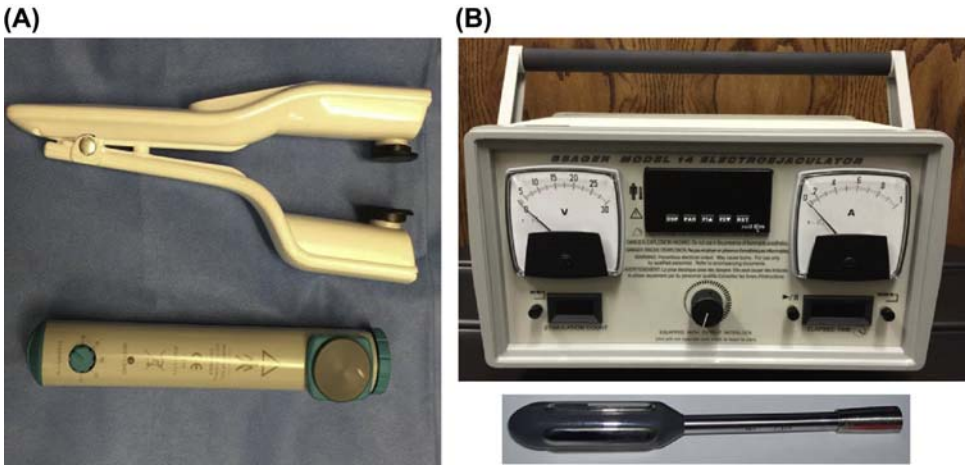

FIGURE 71.5 Instruments to collect semen from patients with spinal cord injury. (A) Penile vibratory stimulators including FertiCare (bottom device) and Viberect X3 (top device). These devices can be placed on the dorsum or frenulum of the penis, or both. (B). Seager Electroejaculator and its transrectal probe. The probe is used to deliver electrical current directly to the prostate and seminal vesicles, resulting in ejaculation. The harvested sperm can be used for either intrauterine insemination or in vitro fertilization.

PENIS

Penile Reconstruction

Congenital and acquired genitourinary tract anomalies of the penis require surgical reconstruction. Lack of sufficient normal autologous tissue is the major limitation in many surgical procedures for different conditions such as ambiguous genitalia, epispadias, hypospadias, micropenis, aphallia, severe chordee, impotence, female to male genital reassignment, and traumatic or iatrogenic penile defects [76–78]. The possibility of developing tissue composed of corporal cells in vivo has also been investigated [79]. Human corporal smooth muscle cells (SMC) and endothelial cells (ECs) were seeded on biodegradable polymer scaffolds at concentrations of 20×10^6 and 10×10^6 cells/cm^3, respectively. These constructs were then implanted in the subcutaneous space of nude mice [79]. Histological evaluation showed the formation of vascularized corpus cavernosum composed of these two cell types 28 and 42 days after implantation [79]. To achieve a 3D corporal structure, naturally derived polymer scaffold from rabbit corporal tissue was used combined with primary human cavernosal SMC and ECs [80]. Engineered corporal tissues were maintained in culture for up to 4 weeks and then implanted into nude mice [80]. The matrices showed stable collagen concentration 8 weeks after implantation [80]. Immunocytochemical studies using α-actin and factor VIII antibodies confirmed the presence of corporal SMC and EC in vivo [80]. Organ bath studies also showed that the cell-seeded corporal tissue matrices responded to electrical field stimulation [80]. In a follow-up study, structural parameters were evaluated up to 6 months after implantation in a rabbit penile defect model [81], in which the engineered corporal bodies showed structural integrity on cavernosography. The presence of sperm after copulation was confirmed in rabbits with the engineered corpora [81]. In all of these studies, it was shown that a short segment of the penile corporal body (approximately one-third of the penile corpora) can be replaced using naturally derived collagen matrices with autologous cells [79–81]. To improve the results of previous studies, engineering of the entire length of both penile corpora was attempted [82]. In another study, the matrices were seeded with the autologous corporal SMC and EC, using a novel multistep cell seeding protocol [83], and the cell seeded matrices were used to replace the entire pendular penile corpora in 12 male rabbits [82]. At 1, 3, and 6 months after implantation of the engineered corpora, the rate of sperm presentation on vaginal swabs and pregnancy rates of mated female rabbits was 83% and 30%, respectively. Untreated rabbits showed no evidence of intravaginal sperm and no pregnancies occurred [82]. These studies demonstrate that penile corpora cavernosa tissue can be engineered and transplanted to restore function.

Penile Transplantation

As an option for penile reconstruction, penile transplantation has been considered, especially after near-complete penile loss. So far, three allogenic penile transplantations have been performed worldwide. The first was performed

successfully in China in 2006; however, it was removed after 2 weeks because of the recipient and his wife experienced severe psychological problems [84,85]. The second case was described in 2014 and was performed in South Africa. The surgery was complicated by a reintervention after 4 days to remove a thrombus from the anastomosis and another reintervention for hematoma drainage. However, the patient reported normal sexual and urinary function 3.5 months after transplantation [86]. The third case was the first US-based penile transplantation and was performed in a patient with penile cancer [87]. The requirement of lifelong multidrug immunosuppression with the risk for serious side effects remains a limiting factor for the widespread clinical application of penile transplantation [88].

Stem Cell Therapy for Erectile Dysfunction

ED is defined as the consistent or recurrent inability to achieve or maintain an erection sufficient for sexual activity [89]. Interest is increasing in recruiting endogenous or exogenous stem cells to treat ED [90]. It is generally believed that tissue-specific stem cells exist in most postnatal tissues. Two types of foreskin stem cells have been isolated , including skin-derived progenitors [91] and MSCs [92]. In a rat study using 5-ethynyl-2′-deoxyuridine labeling, the presence of stem and progenitor cells was shown. These cells, mainly distributed within the subtunic and perisinusoidal space of penis, are defined as subtunic penile progenitor cells and perisinusoidal penile progenitor cells. These cells expressed c-kit, A2B5, and proliferating cell nuclear antigen [93]. It is expected that reactivation of endogenous stem cell potential might rejuvenate damaged erectile function [94]. Human MSCs have been isolated from a large number of adult tissues (bone marrow, adipose tissue, skeletal muscle, etc.). Adipose tissue, which is a rich and easily obtainable source of MSC, has been of particular interest in treating ED [95]. MSCs express low levels of major histocompatibility complex (MHC) class I and do not express MHC class II molecules; thus, they are minimally immunogenic. Therefore, they can be used from either autologous or allogenic sources [96]. Isolated MSCs from rat paratesticular fat tissues were injected in a bilateral cavernous nerve injury model. One month after injection, measurement of intracavernous pressure revealed significant improvement in ED [97]. All of these observations demonstrate how stem cell treatment is a promising tool to restore normal erectile function, but these applications need further development for future clinical trials.

CONCLUSION

Regenerative medicine is a branch of translational research in tissue engineering and molecular biology that deals with the process of replacing, engineering, or regenerating human cells, tissues, or organs to restore or establish the normal function of tissues and organs. Most of the effort toward engineering male reproductive tissues has occurred in animal models. The safety of engineered reproductive tissues is usually more critical than other organs because in many cases, more than just recipients are involved. The health of offspring as well as the patient's partner should be considered in any future clinical trials. Regenerative medicine has opened new avenues to treat disorders of male reproductive system affecting fertility and sexual life.

Acknowledgment

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CHAPTER

72

Regenerative Medicine of the Bladder

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INTRODUCTION

The urinary bladder is a hollow muscular organ lined with urothelium or transitional epithelium. The main source of force, the detrusor muscle, is a layer of the urinary bladder wall made of smooth muscle fibers that is arranged in spiral, longitudinal, and circular bundles. The smooth muscle tissues can be extended to store the urine and then completely empty the bladder. As a passive barrier, the urothelium does not generate mucus. This internal lining of the bladder wall, which is composed of urothelial tissue and lamina propria, is believed to regulate some aspects of overall bladder physiology in response to stimuli such as distension during filling [1].

The urinary bladder functions as a storage vessel for urine to delay the frequency of urination. It is one of the most elastic organs of the body and can increase its volume greatly to accommodate up to 800 mL of urine at maximum capacity in adults. Transitional epithelium, elastic fibers, and visceral muscle tissue in the walls of the urinary bladder contribute to its distensibility and elasticity, allowing it to extend easily and return to its original size several times each day.

Congenital disorders, cancer, trauma, inflammation, and other conditions of the bladder can damage its histologic structure or cause complete loss of function. Both situations usually necessitate eventual reconstruction or replacement of the damaged tissue. Although patients with diseased or injured organs can be treated with transplanted organs, there is a severe shortage of donor organs that worsens yearly as the population ages and new cases of organ failure increase. Lower urinary tract reconstruction has been performed with autogenic nonurologic tissues such as gastrointestinal segments, mucosa from multiple body sites, homologous tissues from a donor (cadaver or living donor kidney), and heterologous tissues or substances (e.g., bovine collagen) [2]. All of these materials may cause a series of complications after surgery because the implanted tissue is rejected or inherently different functional parameters may cause a mismatch in the system. For example, replacement of bladder tissue with gastrointestinal segments can be problematic because the urothelium normally excretes proteins [3] whereas gastrointestinal tissue generally absorbs them. This mismatched state can lead to metabolic complications in addition to infection and other issues [4]. Therefore, replacement of lost or deficient urologic tissues with functionally equivalent ones would improve the outcome of reconstructive surgery in the genitourinary system. This goal may soon be achievable with the use of tissue engineering techniques.

Tissue regeneration uses the principles of cell biology, biomaterials science, bioactive factors, and biomedical engineering to develop biologic substitutes that can restore and maintain the normal function of damaged or lost tissues and organs. Tissue engineering involves using natural or synthetic matrices, often termed scaffolds, that encourage the body's natural ability to repair itself and assist in determining the orientation and direction of new tissue growth. Often, tissue engineering uses a combination of these techniques. For example, matrices seeded with cells can be implanted into the body to encourage the growth or regeneration of functional tissue. These cells, materials, and bioactive factors all could be called "biomaterials."

Scientists have attempted to grow native and stem cells, engineer tissues, and design treatment modalities using regenerative medicine techniques for the urinary tract system. This article reviews progress in bladder reconstruction using tissue engineering technology.

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CELL SOURCES

Bladder and Ureter Cells

Although implanted tissue can be heterologous, allogeneic, or autologous in experimental animal models, only autologous cells are used in the clinic setting [5] to avoid graft rejection and the long-term use of immunosuppressive medications after allogeneic transplantation. Autologous somatic cells obtained from the bladder or ureters [6] are often used for tissue engineered bladders. The donor tissue is dissociated into individual urothelial and smooth muscle cells (SMCs), which are expanded in culture, attached to a support matrix, and then implanted back into the host. Ideally, this approach allows lost bladder tissue function to be restored or replaced with limited complications [7–10]. However, suitable bladder cells from the patient for this purpose are sometimes limited or unobtainable because of bladder exstrophy, malignancy, or other reasons.

Stem Cell Sources

Adult stem cells could be a suitable alternative to bladder cells. They are an appealing cell source for tissue regeneration, in part because of their self-renewal, long-term expansion in vitro, and differentiation potential. Three types of cell sources have been used for bladder regeneration in experiment models: autologous cells (i.e., urothelial cells (UCs) and smooth muscle cells (SMCs), urothelial progenitor cells [11,12], bone marrow-derived stromal cells (BMSCs) [13,14], adipose stem cells (ASCs) [15,16], urine-derived stem cells (USCs) [17–20], hair follicle stem cells [21,22]); allogeneic cells: (such as UC+SMC [23], and MSCs); xenogenous cells: (such as amniotic fluid-derived cells (AFCs) [24], dental pulp stem cells [25], hBMSCs+HSPCs [14], and genetically modified hBMSCs [26]). Different types of stem cells used for urinary bladder reconstruction are listed in (Table 72.1)

Adult stem cells avoid some ethical issues associated with embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). They do not transdifferentiate into a malignant phenotype; thus, there is a diminished risk for teratoma formation or immunogenicity after the cells are implanted in vivo. In addition, these cells possess paracrine effects and immunomodulatory properties, are immune-privileged (i.e., tolerate the introduction of antigens without eliciting an inflammatory immune response), and have a low immunogenic potential [27]. Adult stem cells, especially mesenchymal stem cells (MSCs) and hematopoietic stem cells, are well-investigated cell types in stem cell biology [28]. Adult stem cell research remains an area of intense study, because their potential for therapy may be applicable to various degenerative disorders. Many other types of adult stem cells have been identified in organs all over the body and are thought to serve as primary repair entities for their corresponding organs. The discovery of such tissue-specific progenitors has opened up new avenues for research. Adult stem cell populations have been found in many adult tissues other than the bone marrow, skin [29] and adipose tissue [30], including those traditionally considered a postmitotic organ, such as brain [31], heart [32,33], and urine [34].

ESCs or iPSCs exhibit two remarkable properties: the ability to proliferate in an undifferentiated but still pluripotent state (self-renewal) and the ability to differentiate into different specialized cell types [35]. These cells have been maintained in the undifferentiated state for at least 80 passages and 200 population doublings in vitro when grown using published protocols [36,37]. In addition, human ESCs differentiate into cells from all three embryonic germ layers in vitro. However, these cells are banned for many clinical applications because of risks for immunogenicity and teratoma in addition to ethical dilemmas associated with manipulating embryos in culture.

Although the entire urinary tract system is responsible for excreting ions, metabolic products, and toxic wastes from the body, it is a closed system with a germ-free environment, unlike the digestive system, which is open and does not have sterile conditions. Some living cells in urine can survive for a few hours within the sterile urinary system. Stem cells exist in human voided urine and urine drained from the upper urinary tract. These cells, termed USCs [34], possess stem cell characteristics with clonogenicity, cell growth pattern, robust proliferative potential, and multipotential differentiation. These cells can be obtained using simple, safe, noninvasive, and low-cost procedures, which avoids the adverse events associated with obtaining cells from other sources. A single USC can expand to a large population with 60–70 population doublings [38,39]. When seeded onto a scaffold and subcutaneously implanted into nude mice, multilayered tissue-like structures formed consisting of urothelium and smooth muscle. In addition, USCs differentiated into endothelial, osteogenic, chondrogenic, adipogenic, skeletal myogenic, and neurogenic lineages. However, within the 3 months of study, they did not form teratomas despite telomerase activity. USCs have been used in cell-based therapies and urogenital tissue engineering applications, including urethral tissue [39,40], bladder reconstruction [20], kidney and urethral sphincter [41,42], vesicoureteral reflux [41], and erectile dysfunction [43,44].

TABLE 72.1 Different Sources of Stem Cells for Bladder Regeneration

	MSCs	Primitive Stem Cells	USCs	ESCs/iPSCs	Somatic Cells
Cell types	BMSCs [13,14], adipose stem cells [15,16], Urothelial progenitor cells [11,12], Hair follicle stem cells [21,22] Dental pulp stem cells [25], hBMSCs plus hematopoietic stem and progenitor cells [14] Genetically modified human BMSCs [26]	Amniotic fluid-derived cells [24,48] or placenta-derived stem cells	USCs [17–20],		UC plus SMC [23]
Self-renewal and expanded capability	Limited, PD ~30	High, PD	High, PD 60–70	Very high, PD >200	Limited, PD <30
Multilineage differentiation capability	Multipotent but mainly limited within mesodermal cell lineages	Yes	Multipotent differentiation potential	Pluripotent (can form all lineages)	No
Urothelial, endothelial, or myogenic differentiation capability	Very low	High	High	Low	No
TA/Telomere length	Cannot be detected	Yes	≤75% USC clones possess TA and relative long telomere	Possess TA and long telomere	No
Harvest approach	Invasive, relatively hard to harvest BMSCs at senior level	Aspirate from amniotic fluid	Noninvasive, simple, cost-low, safe	Invasive for iPSCs	Invasive
Pure stem cell isolation	Hard to isolate pure stem cells	Easy	Very easy	Easy	No
Number of stem cells harvested	1 MSC/10^4 BMSCs at newborn baby, 1 MSC/10^6		100–140 USC clones/ 24 h urine in adult		No
Rejection after implanted in vivo	Autogenous or allogeneic	Allogeneic	No rejection	Likely to be rejected if donor is unmatched	No rejection if autogenous cells are used
Oncogenic potential	No reported	No reported	No reported	Yes, harbors disease-causing genes of donor	No reported

AFCs, amniotic fluid-derived cells; *BMSCs*, bone marrow stromal cells; *ESCs*, ethical issues associated with embryonic cells; *iPSCs*, induced pluripotent stem cells; *MSCs*, mesenchymal stem cells; *PD*, population doubling; *SMC*, smooth muscle cell; *TA*, telomerase activity; *USCs*, urine-derived stem cells; *UCs*, urothelial cells.

In chronic bladder diseases, USCs might be a good cell source for bladder tissue regeneration because cells from the upper urinary tract are normal. In treatment of end-stage bladder diseases or muscle-invasive bladder cancer, using engineered bladder tissue with USCs would be superior to bladder reconstruction using intestinal segments. Intestinal segments used for bladder reconstruction appear to be at increased risk for malignancy, particularly adenocarcinoma, because of histologic changes in the intestinal mucosa after long-term exposure to urine. Studies showed that all children with neurogenic bladder disease are at increased risk for bladder cancer regardless of exposure to intestine [45]. Bowel tissue also increases the risk for complications such as stone formation and excess mucous secretion [46]. Harvesting USCs from patients who already have a nephrostomy tube in place would be a simple, noninvasive, and low-cost approach to obtaining cells for engineering bladder tissue.

Alternate sources of stem cells are the amniotic fluid and placenta. These contain multiple partially differentiated cell types derived from the developing fetus [47,48]. Stem cell populations have been isolated from these sources and express embryonic and adult stem cell markers, but they do not form teratomas. The cells are multipotent and can differentiate into cells from all three germ layers. In addition, such cells have high replicative potential and could be stored for future self-use without the risks for rejection and without ethical concerns.

Mechanism of Cell Therapy

Cell Expansion

Fetal stem cells, such as amniotic fluid and primitive stem cells (such as placenta-derived stem cells), showed promise for future clinical applications [47,48]. These cells can develop into cells from the endoderm, mesoderm, and ectoderm and can be maintained for over 250 population doubling (PD). Long telomeres are retained and a normal karyotype without tumorigenicity is observed in vivo [49]. Adult stem cells have been successfully isolated from various types of tissues. These cells usually reach a PD rate of 20–40 in 10 passages [19].

USCs can generate large numbers of cells in a single clone [20,39]. Around 100–140 USC clones can be formed from 24-h urine collection from one individual [50]. Up to 75% of these cells are highly proliferative owing to their relatively higher telomerase activity (USC-TA^{+}) and longer telomeres compared with BMSCs [20]. USCs are reported to have a PD rate of 60–70 for up to 20 passages, whereas other USCs without telomerase activity can be maintained for 8–10 passages with 34 population doublings. Based on this ratio of cells and urine volume, two urine samples containing 20–30 USC clones could potentially yield at least 1.5×10^9 USCs at the end of passage 4 within 4–5 weeks [5,38].

Isolation of USCs is a separation- and digestion-free procedure. Urine samples are simply centrifuged and cells are seeded in mixed media composed of keratinocyte serum-free medium and embryonic fibroblast medium at a 1:1 ratio [34]. Expanded USCs are a relatively homogeneous population and require only 2–5% serum to be maintained in vitro; by contrast, most MSCs require 10–20% serum [20]. When cells collected from voided urine are cultured in USC culture media, only USCs tend to attach to the culture container and continuously expand in culture [9]. This quick, easy, and economical process for USC isolation may also facilitate their large-scale expansion for potential clinical trials.

Multipotentiality

During tissue repair, stem cells accelerate tissue repair and regeneration in various ways (Table 72.2). Although ESCs and iPSCs are pluripotent and can differentiate into multiple specialized cell types [35], their differentiation capability into urologic-specific cell types is relatively low [19]. Under appropriate conditions, BMSCs can be successfully induced into cells with bladder SMC characteristics, both in vitro and in multiple animal models [51–53]. Induced BMSCs proliferate at a rate similar to bladder SMCs and possess a similar histologic appearance and contractile phenotype. However, only 5–10% of cells in BMSCs can be induced into urothelial-like cells with specific lineage marker expression [53–58]. One reason for this could be their relative scarcity; only an estimated 1 in $1–1.5 \times 10^4$ cells in bone marrow are BMSCs [59].

Although the effects of aging on MSCs remain controversial, some studies show an age-related decline in stromal vascular fraction number, proliferation rate, longevity, differentiation potential, and MSC immunophenotype expression in BMSCs from older compared with younger donors [60–64]. This question becomes greatly important because the aging population is increasing, and thus the prevalence of urologic diseases is also increasing. Considering the large quantity of MSCs required for clinical applications (up to 10^9), especially for bladder reconstruction, it is critical to find a stable cell source with potent MSCs [5].

TABLE 72.2 Multiple Modes of Action Assigned to Adult Stem Cells

Multiple Functions of Stem Cells	Outcomes and Potential Applications	References
Multipotent differentiation	Osteocytes, chondrocytes, adipocytes, myocytes, epithelial and endothelial cells for cell replacement	Bharadwaj et al. [20,38]
Secretion of trophic factors	Recruitment resident cells to tissue repair	Liu et al. [119]
Secretion of extracellular matrix	Prompt cell proliferation, rejuvenation, and differentiation of bone marrow-derived stem cells	Pei et al. [120]
Immunomodulatory and antiinflammatory	Inhibited T- and B-cell proliferation to decrease fibrosis	Wu et al. [70]
Gene delivery via angiogenic growth factor gene transfection	Soluble factor gene therapy to accelerate tissue regeneration at site of chronic injury with extensive scarring	Liu et al. and Ouyan et al. [43,121,122].

Unlike many other cell lineages, USCs can efficiently differentiate into urologic tissue-specific cell lineages, including UCs, SMCs, and ECs. They have higher differentiation frequency into UCs than BMSCs. Under the same induction conditions for 7 days, up to 60−70% USCs are induced into a uroepithelial lineage, compared with only 5% for BMSCs [20,65]. When induction extended to 14 days, up to 90% USCs were differentiated. Cells develop a cobblestone-like morphology and express both urothelial-specific cell markers (uroplakin-III and uroplakin-Ia) and generic epithelial cell markers (CK7 and AE1/AE3). Differentiated USCs also present tight junction markers (ZO-1, E-cadherin, and cingulin) in a dose-dependent and time-dependent manner [20]. Those cells display enhanced barrier function with at least a 60% decrease in leakage compared with noninduced cells [20].

Urothelially differentiated USCs were seeded on a porcine small intestinal submucosal (SIS) scaffold and cultured in vitro for 14 days before implantation into nude mice. Constructs generated stratified layers in vivo and neotissue expressed urothelial-specific cell markers (uroplakin-III and uroplakin-Ia). USCs have differentiation potency into SMCs comparable to that of BMSCs [53]. Up to 80% of induced USCs express early smooth muscle differentiation markers (α-smooth muscle actin and calponin), contractile SMC markers (desmin and myosin), and smooth muscle-specific marker (smoothelin) after 14 days' induction with smooth muscle differentiation media in vitro [20]. Cells exhibit rapid lattice contraction as mature SMCs. When implanted in vivo on a porcine SIS scaffold, those cells formed multiple layers of SMCs beneath UC layers and presented SMC markers (desmin, myosin, and α-smooth muscle actin). USCs are also capable of endothelial differentiation with barrier function. EC-induced USCs display vessel-like structures on a solidified Matrigel surface after in vitro induction. Those cells express EC-specific genes (von Willebrand factor [vWF], and CD31), proteins (CD31, vWF, kinase insert domain receptor, fms-like tyrosine kinase-1, and endothelial nitric oxide synthase), and tight endothelial junction marker (VE-cadherin). When implanted subcutaneously into athymic mice, EC-induced USCs effectively form neovessel structures [66].

Paracrine Effects and Immunomodulatory Properties

Stem cells have two important roles in tissue regeneration. First, they can directly replace diseased cells by engrafting, cell fusion, and differentiating into the required host cell type (for example, bone marrow transplant and cell therapy for myocardial ischemia). Second, grafted cells stimulate the host's cells to repair the injured tissue without the donor cells contributing directly to form the new tissue. This happens because the grafted cells secrete factors that signal the host's cells to change their biological behavior and the microenvironment. This signaling from one cell to another is called a paracrine effect.

Adult stem cells also can invoke a paracrine response [67]. The implanted stem cells secrete factors that promote angiogenesis and protect against apoptosis, fibrosis, and inflammation [13,68,69], which prompts the host's cells to repair the tissue themselves. Humans and most mammals have a wound repair mechanism that on its own can only

deal with small wounds, but not for large wounds. Large wound is often remodeled by scar tissue. However, human tissue still possesses regenerative potential once it receives the appropriate signals to initiate internal tissue regeneration and repair provided by paracrine effects of the grafted stem cells.

Although stem cells have a short life span (1−3 weeks) after implantation, they have long-term effects on tissue repair. For example, they appear to be important for initiating tissue regeneration but are expendable once the patient's cells are activated. Paracrine effects are amplified once the grafted cells are attracted to injured tissues. Cells within the damaged tissues often secrete cytokines, regulatory factors that act as mediators to generate an immune response that attracts grafted cells. Sequentially, the grafted cells secrete their own cocktail of proteins that stimulate the host's stem cells and inhabit inflammation and oxidative stress, protect against fibrosis, promote resident cell proliferation, and increase vascularization and blood flow into the injured areas.

In addition, paracrine effects of adult stem cells can reduce immune response and possess immunoregulatory properties. For example, regulatory T cells have an important role in inducing peripheral tolerance, inhibiting proinflammatory immune responses, and decreasing immune reactions. To test the immunomodulatory effects of USCs, our studies demonstrated that USCs can inhibit the proliferation of peripheral blood mononuclear cells (PBMNCs) (T and B cells), and secretion of interleukin (IL)-6 and IL-8 [70]. In the specific experiment, the mononuclear cells usually proliferate when mixed with other somatic cells owing to immune stimulation. In contrast, PBMNC concentrations in USC wells are much lower than in somatic cells, even BMSC culture wells [70]. Colorimetric enzyme-linked immunosorbent assays showed less 5-bromo-2′-deoxyuridine labeled in the USC PBMNC-mixed culture wells and more in BMSC culture wells. As costimulatory molecules, CD80 and CD86 initiate and modulate T-cell immune response. Both CD80 and CD86 expressed on the surface of antigen-presenting cells interact with cytotoxic T-lymphocyte antigen-4 expressed on activated T cells and mediate critical T-cell inhibitory signals. About 3% of the BMSCs were positive for CD80 (versus 1.05% of USCs) and 1.3% of the BMSCs were positive for CD86 (versus 0.55% of USCs). Human cytokine release arrays showed that IL-6 and IL-8 concentrations were elevated after stimulation by PBMNCs in USC supernatant, higher than BMSC supernatant. Thus, it indicates that IL-6 and IL-8 might be the main immunomodulatory cytokines to target in future studies aimed at preventing and treating bladder abnormalities that accompany diabetes, other immune system disorders, or rejection of transplanted organs.

BIODEGRADABLE BIOMATERIALS

A biological scaffold tailored to stem cells is central to mimicking the function of the extracellular matrix (ECM) in tissue engineering. The ECM provides structural support and a physical environment so that cells can attach, proliferate, migrate, differentiate, and function [71]. It confers mechanical properties to tissues and delivers bioactive cues for regulating activities of residing cells; it also provides a dynamic environment for vascularization and new tissue formation. Scaffolds can be designed to stimulate and direct tissue formation to replace portions of tissues or whole tissue structures. The material would possess appropriate porosity and microporosity (interconnectivity between pores) to expedite cell attachment, migration, penetration, differentiation, tissue growth, and integration. The ideal type of cell replacement should be composed of materials with similar physical and mechanical properties as the native tissue and should degrade at the same rate as the new tissue is generated. Porosity should allow nutrient transfer and cell adhesion without compromising mechanical strength. Two categories of scaffolds designed to carry cells include synthetic scaffolds and natural collagen matrix for bladder regeneration (Table 72.3).

Synthetic Scaffolds

Various porous structures composed of natural or synthetic biodegradable and biocompatible materials have been used as scaffold carriers. Some biomaterials have been approved by the US Food and Drug Administration (FDA) for use in medical devices in humans. These include polyesters of naturally occurring α-hydroxy acids such as polyglycolic acid (PGA), polylactic acid, poly(lactic-*co*-glycolic acid), and polycaprolactone. Metabolites formed after degradation of these materials have been confirmed to be nontoxic and are eventually eliminated from the body as carbon dioxide and water [72]. Because these polymers are thermoplastics, they can easily be formed into a three-dimensional (3D) scaffold with the desired microstructure, gross shape, and dimensions by various techniques, including molding, extrusion [73], solvent casting [74], phase separation techniques, and gas foaming techniques [75]. In addition, synthetic polymers can be manufactured on a large scale using various

TABLE 72.3 Summary of Advantages and Limitations of Biodegradable Materials for Bladder Regeneration

	Advantages	Limitations
SYNTHETIC SCAFFOLDS		
• PGA/PLGA • PLLA • Collagen-fibrin hybrid scaffold with IGF1 [123] • Acellular collagen-heparin scaffolds with growth factors [124] • VEGF-loaded nanoparticle-modified BAMAs [125] • PGA with PGA–chitosan "sandwich" graft [126]	• Renewable, sustainable, biodegradable • Safe for use, nontoxic, free-immunogenic, free-carcinogenic, free-thrombogenic • Ease of manufacturability • Reasonable cost • Durable and storage stability • Process ability: fine complex architectures such as porosity rate, pore size can be generated; highly controllable • Consistent products	• Take time to be degraded • Poor biocompatibility • potential toxic degradation of by-products • Lacks specific integrin-binding site
BIOLOGICAL SCAFFOLDS		
Silk fibroin [23,127,128], or BAMG-SF matrix [128] SIS [129–131], BSM BAMG [15,24,132] or UBM [133] Decellularized colon matrix [134] Matrix with hydrogel (such as HA-PLGA-modified SIS [135])	• Unlimited source biomaterials • Easy to produce • Most allogenic or xenogeneic antigen contents can be removed after decellularization • Native architecture is preserved • Growth factors and cytokines bound with matrix remain • Integrin-binding sites are retained • Easy to suture to bladder tissue	• Variability depending on the source of biomaterial • Antigenicity • Weak biomechanical properties • Not suitable for subtotal cytoplasty • Take time to produce in the laboratory

BAMA-SF, bladder acellular matrix graft-silk fibroin; *BAMG*, bladder acellular matrix grafts; *BSM*, bladder submucosa membrane; *HA-PLGA*, hydroxyapatite-poly(lactide-*co*-glycolide). *IGF*1, insulin-like growth factor 1; *PGA/PLGA*, polyglycolic acid/polylactic-*co*-glycolic acid; *PLLA*, poly-L-lactic acid; *SIS*, small intestine submucosa; *UBM*, urinary bladder matrix; *VEGF*, vascular endothelial growth factor.

techniques including electrospinning [76], phase separation, gas foaming, particulate leaching, inkjet printing, and chemical cross-linking. Strength, degradation rate, and microstructure may be adjusted during manufacturing. Scaffolds can be made in different shapes and porosity to facilitate cell engraftment, or they can be further modified by incorporation, surface adsorption, or chemical attachment of bioactive factors. However, a drawback of synthetic polymers is the lack of biologic recognition. To incorporate cell recognition domains into these materials, copolymers with amino acids have been synthesized [77]. Other biodegradable synthetic polymers, including poly(anhydrides) and poly(orthoesters), can be used to fabricate scaffolds with controlled properties [78].

Biodegradable Properties

Biodegradable biomaterials in tissues of the urinary tract system function as an artificial ECM to replace biologic and mechanical functions of native ECM. These biomaterials facilitate localization and delivery of cells or bioactive factors (e.g., cell adhesion peptides, growth factors) to desired sites in the body and define a 3D space for the formation of new tissues with appropriate structures. They are also a guide for the development of new tissues with appropriate function. Direct injection of cell suspensions without such matrices has been used in some cases [79]. However, without this scaffold function, localization of transplanted cells is difficult to control.

For cell-based tissue engineering, expanded cells are seeded onto a scaffold synthesized with the appropriate material. Because most mammalian cell types are anchorage-dependent and will die if no cell adhesion substrate is available, biomaterials provide such a substrate capable of delivering cells to specific sites with high loading efficiency. Biomaterials can also provide mechanical support against in vivo forces, maintaining the predefined 3D structure during tissue development. Furthermore, bioactive signals such as cell-adhesion peptides and growth factors, can be loaded to regulate cellular function. Generally, two classes of biomaterials are used for engineering of genitourinary tissues: synthetic polymers and acellular tissue matrices. Although synthetic polymers can be produced on a large scale with controlled properties of strength, degradation rate, and microstructure, naturally derived materials and acellular tissue matrices have the potential advantage of biologic recognition, which can lessen host-versus-graft reactions.

Limitations of nutrient and gas exchange restrict tissue engineered implants to a volume of approximately 3 mm^3. Therefore, to engineer large complex tissues and organs, vascularization of the regenerating tissue is essential. Three approaches have been used to encourage the vascularization of bioengineered tissue. First, incorporating angiogenic factors into bioengineered tissue can attract host capillaries and enhance neovascularization. Second, some studies have investigated the effects of seeding endothelial cells with other cell types in the bioengineered tissue [69]. Finally, vascularizing the matrix before cell seeding has been attempted. There are many obstacles to overcome before large tissue engineered solid organs are produced, but developments may provide important knowledge and essential materials to accomplish this goal.

Porosity

Recellularization of biological materials (scaffold) with cell-seeded technology provides a promising option in tissue regeneration. The scaffold needs to have a 3D structure with high porosity but also maintain normal tensile strength. A 3D scaffold with higher porosity and relative larger pore size (50−200 μm) promotes cell proliferation, migration, and infiltration into the matrix and appears to allow abundant cell loading onto the scaffold, thereby promoting in vivo tissue regeneration [80−82]. Treatment with 5% peracetic acid led to high porosity on the surface of the matrix with less cellular material retained; about 75% of normal tensile strength remained. Cells penetrated deeper into the lamina propria of the matrix compared with untreated matrix [83].

Natural Collagen Matrix

Naturally occurring matrix materials may also function as 3D scaffolds in tissue engineering and regenerative medicine, including both decellularized natural matrix and matrix produced from naturally extracted polymers. Natural materials are widely used in tissue regeneration such as bladder submucosa (BSM) and SIS, and naturally derived materials such as collagen, silk fibroin (SF), and alginate. As the most abundant structural protein in the body, collagen is an FDA-approved biocompatible material for various medical uses. Natural decellularized ECM retains tissue-specific architecture and provides various biological and physical material properties specified by the nature of the original tissue. Such matrix materials also share highly conserved matrix proteins among species, such as collagen, laminin, and fibronectins, which suggests that they could be nonimmunogenic and attractive for the purposes of recellularization and tissue integration.

Under certain circumstances, UCs and SMCs differentiated from adult stem cells can form multiple uniform layers on porcine decellularized SIS scaffold in vitro and in vivo, which shows the potential of this 3D cell/matrix to develop into a multilayer mucosal structure similar to native urinary tract tissue [40]. When seeded with USCs, bacterial cellulose scaffolds represent a promising material for urinary conduits with a multilayered urothelium and cell/matrix infiltration in vitro and in vivo [39]. However, in the context of future clinical applications, decellularized matrix could be contaminated by xenogeneic factors. There is also a risk for incomplete decellularization and residual cell bodies and altered tissue properties owing to complete decellularization and deproteinization [84,85].

Acellular Tissue Matrices

Acellular tissue matrices are collagen-rich matrices prepared by removing cellular components from tissues, often by mechanical and chemical manipulation of a segment of bladder tissue and small intestine [40,86]. Matrices slowly degrade after implantation and are replaced and remodeled by ECM proteins synthesized and secreted by transplanted or ingrowing cells. Acellular tissue matrices support cell ingrowth and regeneration of genitourinary tissues, including urethra and bladder, with no evidence of immunogenic rejection [86]. Because protein structures (e.g., collagen and elastin) in acellular matrices are well-conserved and normally arranged, the mechanical properties of acellular matrices are not significantly different from those of native BSM [87].

Collagen

Collagen is the most abundant and ubiquitous structural protein in the body and is readily purified from both animal and human tissues through enzyme treatment and salt/acid extraction. Collagen exhibits minimal inflammatory and antigenic responses and is approved by the FDA for many types of medical applications, including wound dressings and artificial skin [88]. This material can be processed into various structures such as sponges, fibers, and films [89].

Silk

SF is a protein obtained from *Bombyx mori* cocoons and may have potential uses in low urinary tract reconstruction. SF contains up to 90% of the amino acids glycine, alanine, and serine found in other natural biomaterials. SF has excellent biocompatibility and low inflammatory properties and can be fully degraded by naturally occurring proteolytic enzymes [90]. SF has used as an effective biomaterial for bones [91], cartilage [92], blood vessels [93], peripheral nerves [94], cornea [95], bladder [96], and urethra [97], among other tissues. Silk processing methods enable the construction of films [98], foams [99], hydrogels [100], gel-spun matrices [96], and woven or nonwoven meshes [101]. Electrospinning may also be an appropriate technique for urethral reconstruction with SF because it can create a 3D and highly porous scaffold in a conformation that mimics ECM structure in vivo [76].

Matrix Binding With Growth Factors

Tissue engineering approaches should mimic the in vivo setting by providing a biocompatible scaffold, a cell source of appropriate progeny or type, nutrients and other environmental conditions, and appropriate bioactive factors. Localized delivery of bioactive factors is understood to be effective and necessary in mimicking the natural microenvironment of cells within specific tissues. Delivering biomolecular cues, especially growth factors, in solution is difficult because of their rapid diffusion to extracellular fluids, and growth factors always have a limited half-life in vivo. However, sustained delivery of growth factors is important for clinical applications of tissue engineering.

Vascular endothelial growth factor (VEGF) is an important active protein for inducing angiogenesis in tissue engineering, but it requires a delivery system targeted to a given site. There are several ways in which matrix could binding with growth factors. Native human VEGF fused with a collagen-binding domain (CBD-VEGF) can bind to collagen specifically to exert angiogenesis effects that promote regeneration. CBD-VEGF was constructed by linking a sequence that encodes the collagen-binding domain (TKKTLRT) with VEGF complementary DNA. CBD-VEGF was inserted into one plasmid (pET-28a). The plasmid was transformed into the BL21 strain of *Escherichia coli*. CBD-VEGF could be purified from the protein made from *E. coli*. When collagen was used as a scaffold in tissue engineering to which CBD-VEGF can bind in vitro and in vivo, VEGF could maintain activity [102].

PRECLINICAL MODELS

Tissue Regeneration Models

Two in vivo experimental models have been used for bladder regeneration, including hemi-cystoplasty (in which 40−50% of bladder tissues are removed) and subtotal cystoplasty (in which >75% bladder tissue is removed or trigone-sparing cystectomy is performed) (Table 72.4). Cell-seeded or cell-free seeded scaffolds are capable of increasing bladder volume in small animals after hemi-cystectomy, whereas cell-seeded scaffolds are often designed to replace diseased bladder in a larger animal model after subtotal cystectomy. A rodent model such as rat is often used to test new cell sources or new biomaterials and study histologic changes in the regenerative bladder. Larger animal models are used to evaluate further the alterations in bladder function and volume, and anatomic and histologic structures (Table 72.5), in preparation for clinical applications. Beagles or minipigs are most commonly used larger animal models for these purposes.

Fibrotic Bladder Model

Most in vivo experiment models of tissue engineered bladders are performed in the normal bladder after cystectomy. However, in clinical situations, patients have diseased bladders with fibrosis or chronic inflammation or lack a good blood supply. Thus, a model is needed that mimics disease conditions to test the potential of tissue engineering technology. Several approaches have been used to create a neurogenic bladder dysfunction model [103] or fibrotic bladder model induced by bladder atrophy owing to urinary diversion [104], bladder ischemia [105], or chemical agents [106].

To generate a bladder atrophy model, urinary diversion is performed to cause a rapid decrease in contractile function in adult or fetal animal bladders. Liu et al. [104] developed a urinary diversion model in the rat. Female Sprague-Dawley rats were distributed into age matched control, sham urinary diversion and urinary diversion groups. Each group was subsequently evaluated 1 or 8 weeks after urinary diversion or sham operation. Diversion was done by surgical disconnection of the ureters from the bladder and implantation into the uterine cervix. The results demonstrated that bladder weight decreased in the diversion group. The urinary diversion group had decreased

TABLE 72.4 Experimental Animal Models for Bladder Reconstruction

	Advantages	Challenges
SURGICAL PROCEDURES		
Hemi-cystoplasty	• Evaluation of cell-seeded or nonseeded tissue engineered technology for bladder augmentation • Neobladder often retains contractile function because of original bladder plus regenerated bladder • Histologically, fully regenerated urothelium; partial muscle regeneration and innervation	Stone formation, graft shrinkage
Subtotal cystoplasty	• Assessment of cell-seeded tissue engineering technology for bladder replacement • Acts as urinary reservoir • Regenerated urothelium • Bladder capacity increases at early stages	• High risk of graft collapse or shrinkage and bladder stone formation • Limited innervation, myogenesis and blood supply at center of graft • Bladder volume may decrease over time
ANIMAL MODELS		
Large animals		
Canine [24,132,135,136]; Porcine, i.e., juvenile Yorkshire swine [27,127]; rabbit [23]; sheep [124]; nonhuman primates [13]	• Physiology, anatomy, and histology similar to humans; • Autograft models; • Easy development of subtotal cystoplasty model • Test both synthetic or thicker nature-based collagen scaffold with cells • Easy to perform urodynamic studies • Enough tissue regenerated to perform contractility studies of muscle strips in organ bath	• High cost to purchase and maintain large animals • Not easy to fix urethral catheter after surgery in pigs and nonhuman primates
Small animals		
Athymic rodent [26] and Rat [15,123,128,134]	• Easy to maintain • Well-known histology • Allogeneic and xenogeneic-grafted stem cells not rejected by the host • Economical • Commonly used for hemi-cystoplasty • Dynamically observe bladder regenerative processes at the different time points • Allogeneic or even xenograft models	• Subtotal cystoplasty model not possible • Require thinner scaffold • High risk of stone formation • Requires special fine devices for urodynamic study and images • Cannot observe immune reaction within graft in immunodeficient rodents

intercontractile intervals and voided volumes compared with the control and sham-operated groups. Compliance was decreased in bladders of rats with urinary diversion. Smooth muscle and urothelium were decreased as a proportion of the total bladder cross-sectional area. Collagen increased in rats with either 1 or 8 weeks of urinary diversion versus controls. Urinary diversion caused decreased expression of muscarinic 3 and ligand-gated purinergic 1 receptor but no change in muscarinic 2 or ligand gated purinergic 2 receptors. Urinary diversion causes dysfunctional and abnormal morphometric alterations in the bladder in this model.

In a study by Matsumoto et al. [107], urinary diversion was performed on fetal sheep after 90 days of gestation (term = 147 days) and bladder tissue was obtained 2 weeks later. Bladders from fetal sheep subjected to urinary diversion weighed significantly less than control fetal bladders. Marked reorganization of smooth muscle elements was observed in those with diversion. Carbachol stimulated a tonic contraction whereas field stimulation administered during tonic contraction elicited a phasic relaxation or a biphasic response consisting of an initial relaxation

TABLE 72.5 Criteria for Tissue Engineering Techniques for Bladder Regeneration

PREIMPLANTATION	
Cells	• Characterize cell proliferation capacity, phenotypes, paracrine effects of stem cells, urothelially and myogenically differentiated stem cells or cultured urothelial and smooth muscle cells with immunocytochemical staining; label to monitor fate of grafted cells if needed.
Scaffold	• Determine biocompatible, biodegradable and mechanical properties, porosity, pore size, and bioactive proteins within scaffold (for natural collagen-based extracellular matrix biomaterials)
Cell–biomaterial interaction	• Demonstrate interactions for cell adhesion and infiltration into scaffold using histologic techniques
POSTIMPLANTATION	
Gross examination	• Graft size and hardness, tissue adhesion around organs • Signs of graft contraction, stones, or calcification on bladder lumen side
Bladder function tests	• Bladder volume, pressure, and contraction style with urodynamics • Contractility of regenerated bladder strips assessed with organ bath
Histologic structure	• Cell survival rate • Distribution and life span of labeled graft cells • Entirety of urothelial layers and thickness of muscle layers • Ratio of collagen and muscle in regenerated bladder tissue • Angiogenesis or revascularization and innervation

and then a phasic contraction in control and diverted bladders. Contractile responses of defunctionalized strips to carbachol were significantly less than those of control bladder strips. In brief, urinary diversion in normal fetal sheep resulted in marked bladder atrophy, reduced muscle mass, and decreased bladder contractility.

Both indwelling urethral catheterization and suprapubic catheterization are types of urinary diversion. The former is associated with a risk for urolithiasis, urethral trauma, urethral erosions and/or strictures, bladder fibrosis, epididymitis, orchitis, and bladder cancer. Therefore, in most clinical cases, indwelling urethral catheters are inappropriate for long-term treatment and suprapubic catheters are a better option. However, clinical data [108] showed that long-term indwelling urinary catheterization has similar rates of upper tract damage, vesicoureteral reflux, renal or bladder calculi, and symptomatic urinary tract infections compared with suprapubic catheters. The catheter was changed every 2 weeks, which can prevent the complications of indwelling urethral catheterization. Although a bladder dystrophy model is closer to diseased conditions, it is time-consuming to create such a model. A novel bladder fibrotic model is needed for bladder tissue regeneration studies.

CLINICAL TRIALS

Clinical Translation

Human urothelial and muscle cells can be expanded in vitro, seeded onto polymer scaffolds, and allowed to attach and form sheets of cells. The cell–polymer scaffold can then be implanted in vivo. Histologic analysis indicated that viable cells were able to self-assemble back into their respective tissue types and would retain their native phenotype [109]. These experiments demonstrated for the first time that composite layered tissue engineered structures could be created de novo. Before this study, only nonlayered structures had been created in the field of regenerative medicine.

To determine the effects of implanting engineered tissues in continuity with the urinary tract, animal models of bladder augmentation were used [110]. Partial cystectomies were performed in dogs. The animals were divided into two experimental groups. One group had the bladder augmented with a nonseeded bladder-derived collagen matrix; the second group had the bladder augmented with a cell-seeded construct. The bladders augmented with matrices seeded with cells showed a 100% increase in capacity compared with bladders augmented with cell-free matrices, which showed only a 30% increase in capacity.

Most of the free grafts (without cells) used for bladder replacement in the past were able to show adequate histology in terms of a well-developed urothelial layer, but they were associated with an abnormal muscular layer that varied in terms of its full development. It has been well-established for decades that the bladder is able to regenerate generously over free grafts. Urothelium is associated with a high reparative capacity. Bladder muscle tissue is less

likely to regenerate in a normal fashion. Both urothelial and muscle ingrowth are believed to be initiated from the edges of the normal bladder toward the region of the free graft [111]. Usually, however, contracture or resorption of the graft has been evident. The inflammatory response toward the matrix may contribute to the resorption of the free graft. It was hypothesized that building 3D structure constructs in vitro before implantation would facilitate the eventual terminal differentiation of the cells after implantation in vivo and minimize the inflammatory response toward the matrix, thus avoiding graft contracture and shrinkage. The dog study demonstrated a major difference between matrices used with autologous cells (tissue engineered matrices) and those used without cells [110]. Matrices implanted with cells for bladder augmentation retained most of their implanted diameter, as opposed to matrices implanted without cells for bladder augmentation, in which graft contraction and shrinkage occurred. The histomorphology demonstrated a marked paucity of muscle cells and a more aggressive inflammatory reaction in matrices implanted without cells. Epithelial mesenchymal signaling is important for the differentiation of bladder smooth muscle [112]. The results of initial studies showed that the creation of artificial bladders may be achieved in vivo; however, it could not be determined whether the functional parameters noted were caused by the augmented segment or the intact native bladder tissue. To address the functional parameters of tissue engineered bladders better, an animal model was designed that required a subtotal cystectomy with subsequent replacement with a tissue engineered organ [113].

Cystectomy-only and nonseeded controls maintained average capacities of 22% and 46%, respectively, of preoperative values. An average bladder capacity of 95% of the original precystectomy volume was achieved in the cell-seeded tissue engineered bladder replacements. These findings were confirmed radiographically. The subtotal cystectomy reservoirs that were not reconstructed and the polymer-only reconstructed bladders showed a marked decrease in bladder compliance (10% and 42% total compliance, respectively). The compliance of the cell-seeded tissue engineered bladders showed almost no difference from preoperative values that were measured when the native bladder was present (106%). Histologically, the nonseeded scaffold bladders presented a pattern of normal UCs with a thickened fibrotic submucosa and a thin layer of muscle fibers. The retrieved tissue engineered bladders showed a normal cellular organization consisting of a trilayer of urothelium, submucosa, and muscle. Immunocytochemical analyses confirmed the muscle and urothelial phenotype. S-100 staining indicated the presence of neural structures [113]. These studies, which were performed with PGA-based scaffolds, have been repeated by other investigators and showed similar results in large numbers of animals over the long term [114]. The strategy of using biodegradable scaffolds with cells can be pursued without concerns regarding local or systemic toxicity [115]. However, not all scaffolds perform well if a large portion of the bladder needs replacement. In a study using SIS for subtotal bladder replacement in dogs, both the unseeded and cell-seeded experimental groups showed graft shrinkage and poor results [116]. The type of scaffold used is critical to the success of these technologies. The use of bioreactors, in which mechanical stimulation is started at the time of organ production, has also been proposed as an important parameter for success [117,118].

To evaluate the effect of cell-seeded tissue engineering technology in the bladder regeneration compared with scaffold alone, a group of experimental dogs underwent a trigone-sparing cystectomy and were randomly assigned to one of three groups. One group underwent closure of the trigone without a reconstructive procedure, another underwent reconstruction with a nonseeded bladder-shaped biodegradable scaffold, and the last underwent reconstruction using a bladder-shaped biodegradable scaffold that was seeded with autologous urothelial and SMCs [113]. The cystectomy-only and nonseeded controls maintained average capacities of 22% and 46%, respectively, of preoperative values. An average bladder capacity of 95% of the original precystectomy volume was achieved in the cell-seeded tissue engineered bladder replacements; however, the subtotal cystectomy reservoirs that were not reconstructed and the polymer-only reconstructed bladders showed a marked decrease in bladder compliance (10% and 42%, respectively, of total compliance). The compliance of the cell-seeded tissue engineered bladders was almost no different from preoperative values (106%). Histologically, the nonseeded scaffold bladders presented a pattern of normal UCs with a thickened fibrotic submucosa and a thin layer of muscle fibers. The retrieved tissue engineered bladders showed normal cellular organization consisting of a trilayer of urothelium, submucosa, and muscle [113], indicating the benefit of cell-seeded tissue engineering technology in the bladder reconstruction, compared with the nonseeded tissue engineered bladder.

Clinical Studies

Clinical experience involving engineered bladder tissue for cystoplasty reconstruction started in 1998. A small pilot study of seven patients was reported [5] using a collagen scaffold seeded with cells with or without omentum

coverage or a combined PGA−collagen scaffold seeded with cells and omental coverage. Patients reconstructed with the engineered bladder tissue created with the PGA−collagen cell seeded scaffolds showed increased compliance, decreased end-filling pressures, increased capacities, and longer dry periods over time [5]. It is clear from this experience that the engineered bladders continued to improve with time, mirroring their continued development. Although this report was promising in terms of showing that engineered tissues can be implanted safely, it is just a start in terms of accomplishing the goal of engineering fully functional bladders. This was a limited clinical experience; the technology is not yet ready for wide dissemination because further experimental and clinical studies are required. FDA phase II studies have been completed.

CONCLUSION

We reviewed experimental and clinical data related to bladder regeneration, highlighting the use of different stem cell types and various biomaterials. Progress suggests that engineered urologic tissues may have wide use for clinical applicability. Assessments of the histologic structure and physiologic function of the urinary bladder can better elucidate mechanisms responsible for functional tissue engineered bladders. Well-established in vitro and in vivo models are available for experimental evaluations of the regenerated bladder, providing invaluable data to predict clinical efficacy.

Standard cell culture studies can define the biological and molecular cues of urothelial, smooth muscle, and endothelial cells or differentiated stem cells. Novel and noninvasive cell sources are needed to improve the regenerative efficacy of urinary bladder further. In addition, 3D construction remains critical to recapitulate the epithelial-stromal microenvironment in bladder regeneration studies. The development and optimization of reliable and reproducible scaffolds with the necessary porosity, biodegradability, flexibility, and firmness are vital for assessing the in vitro and in vivo efficacy of tissue engineered bladders. Biomaterials coated with growth factors are promising tools for bladder regeneration. Therapeutic investigations should be continued with the development of new biomaterials and optimized cell source to improve treatment outcomes for bladder diseases through tissue engineering technology.

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CHAPTER

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Therapeutic Applications: Tissue Engineering of Skin

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INTRODUCTION

There has been an explosion of activity in the field of skin tissue engineering (TE) specifically focused on skin repair and regeneration. A simple online search of the key words "tissue engineering" and "skin," 2007–17, reveals 51,842 publications, 41,780 of which are peer reviewed. Progress has been made in the areas of biomaterials for scaffold generation, the reliable source of cells, and novel approaches to assembly such as three-dimensional (3D) printing. However, skin is much more than a two-layered waterproof construct that provides microbiological protection. Comprehensive skin functioning involves maintaining homeostasis with intimate connectivity with the immune neuroendocrine systems. There is an intimate relationship with the underlying fat of the hypodermis and skin adnexal structures are functionally active [1]. Therefore, a fully integrated vascular and neural network is essential for cellular and neural signaling and driving cutaneous responses to changes in both the host and external environments [2]. The skin adnexal structures remain elusive to large-scale replication although our body of knowledge is building. Furthermore, the challenges of developing a vascularized construct integrating small vessels are such that the rapid or immediate restoration of skin function remains an ambitious goal [3].

Each of us is a self-organizing mass of multiple cell types. From fertilization of the embryo onward, our tissue structures develop until an adult morphology is achieved. At that point, our capacity for self-organization is directed to maintaining that morphology in the face of daily insults and the processes of aging. When a given insult overwhelms our capacity to repair by regeneration, the result is scar repair [4].

We know that tissues retain the variable ability to heal by regeneration [5]. With respect to the skin, in all but trivial injuries the capacity for regeneration is exceeded, triggering cellular mechanisms that result in scar formation. The mechanism of pathology or injury also has an impact on the outcome; for example, burn injury is notorious for developing aggressive scars that compromise the individual functionally, cosmetically, and psychologically [6].

It has been well-described that to heal a wound, we need a source of cells capable of differentiating into the given tissue type and an extracellular matrix (ECM) capable of supporting cell migration, proliferation, and differentiation [7]. We and others have spent considerable resources researching these areas to improve the speed and quality of wound healing to reduce the scar [8]. The question we ask is: "How can we harness the technology of TE of skin to provide a controlled repair to restore the original morphology?"

TE was initially coined by Vacanti in 1990 and can be defined as the application of engineering principles to biological systems [9]. The healing of skin has been the subject of writings as far back as ancient times with the attempts to stimulate healing, protect the surface while healing, and even replace the skin surface [10]. Explorations into using TE principles in skin repair are based on a significant history. With the increasing knowledge and understanding of the skin structure and function along with the developing TE techniques, the question remains, can we provide a regenerative repair avoiding scar?

We know that the skin provides the barrier to the external environment as a dynamic, complex, 3D structure made of cells from all embryological layers. Integral to its functions are the vessels and nerves within the tissue construct. Furthermore, skin is specialized over different body sites, adapting to local functional needs demonstrated by the

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macroscopic differences seen between areas, e.g., the eyelid compared with the palm of the hand. Notable body site differences are seen in the distribution and character of the hypodermis and adipose tissue with an influence on the function and repair potential. The varying capacity of skin to respond to injury is seen with the greater scar potential of sternal and deltoid areas [11].

Thus, as we go forward in developing TE solutions to skin, it is timely to take stock of the skin's functions and interactions. How will skin's changes over the different body sites influence TE needs? Collaboration with specialists in bioinformatics will be essential to understanding the implications of changes in genes, genetic expression, and epigenetics with the resulting phenotypic expression we need to guide the cells and matrix interactions into the tissue constructs [12].

In the therapeutic use of TE, an understanding of the etiology of the skin defect and pathophysiology of the patient will identify what needs to be replicated, rebuilt, and replaced.

Implementation into clinical practice hinges on the TE being problem-driven, providing a practical, timely, cost-effective solution to the clinical problem [13]. TE technologies have been used to produce skin constructs used to test a range of topical preparations and explore toxicology. However, from a clinical viewpoint, TE of skin has developed in response to the clinical need for, e.g., skin repair in major burns [14], chronic ulcers [15], and giant nevi [16]. It is clear that the current reference standard of skin loss, skin grafting, will always leave a scar [17]. The development of TE creates the opportunity to tailor the repair to the defect with the understanding that one solution will not fit all. TE offers innovative solutions providing a spectrum of clinical solutions including:

- strategies to facilitate wound healing in situ by introducing bioactive agents ranging from biomaterials to cell-based therapies, and
- the development of laboratory-based, multilayered tissue constructs including multiple cell types tailored to a specific skin defect.

The development of TE of skin is intimately linked to the vision of facilitating scar-free healing. It has broad implications for after trauma or surgery and in fibrosing pathologies in which the common outcome is a functional compromise owing to the distortion of normal tissue architecture. In the developed world, it is estimated that 100 million patients acquire scars each year as a result of 55 million elective operations and 25 million operations after trauma. Within this number, it is estimated that there are approximately 11 million keloid scars and four million burn scars, 70% of which occur in children [18]. There is a clear opportunity to affect survival and quality of life by engaging in TE solutions.

We are living in a time when science and technology are advancing at an exponential rate. Harnessing the power of that science and technology into clinical practice presents an ever-increasing challenge. We are all aware of the latest breakthroughs holding promise to improve the quality of life, such as genetic engineering facilitating DNA manipulation to improve health. However, the growth of knowledge is possible in controlled systems in which experiments can be designed to investigate a single variable; here lies the greatest challenge: for example, in burn injury research. The design of clinical trials is dogged by the complexities of assessing the extent of injury, the individual's response, and the availability of validated outcome measures.

Clinical practice is a fusion of experience and knowledge with the development of medical subspecialization directed toward a targeted problem-solving approach and has facilitated great advances in clinical care. However, this should not be at the expense of broad general knowledge gaining insight into potential links and facilitating cross-fertilization. It is essential to link the tissue engineer with the clinical specialist to ensure that the opportunities, risks, and benefits of TE skin are understood, to facilitate appropriate clinical trials. By collaboration among disciplines, there are real opportunities for improvements in clinical care translating to improved outcomes for patients [19].

The road from the bench to the bedside is long and navigates the areas of regulation, commercialization, reimbursement, and clinical trial design, to name a few. Also, translational research itself is an area in need of research and audit. The investigation of drivers and barriers to the implementation of TE skin solutions is an area of increasing effort. It is vital to learn from history and understand the current situation to continue developing innovative TE solutions but also innovative solutions within health systems, to move timely translation into clinical practice.

To put the challenges and opportunities into context, this chapter will:

- explore the functions of the skin and injury responses we need to understand to harness the TE technology effectively,
- identify the needs that could be supplied by TE strategies,

- discuss available technologies,
- demonstrate TE skin solutions in clinical practice, and
- look to future challenges in areas with a need for further development.

DEVELOPMENT, ANATOMY, AND FUNCTION OF SKIN

Skin is commonly described as a multilayered physical barrier composed chiefly of the surface cellular epidermis and relatively acellular dermis. Exploring the development, anatomy, and functions of skin demonstrates its complexity and will guide our TE endeavors.

> There is no magician's mantle to compare with the skin in its diverse roles of waterproof, overcoat, sunshade, suit of armour and refrigerator. Sensitive to the touch of a feather, to temperature and pain, withstanding the wear and tear of three score years and ten and executing its own running repairs. *[20]*

During development the initial covering of the embryo is the periderm, which is thought to have a barrier function expressing tight junctions; it interacts with the amniotic fluid, which has surface microvilli. Periderm cells express keratins, which are also associated with migratory epidermal cells seen in wound healing. During the first 6 weeks, the epidermis becomes two-layered, with the outer periderm and the inner developing epidermis. During this time there is no dermis, but a subepidermal cellular layer with deposition of basement membrane type IV collagen and laminin is seen by 5 weeks. By 8 weeks, there is evidence of vascular development. During the embryonic fetal transition stage at 9–10 weeks, several changes are seen:

- epidermal cells express keratins 1 and 10, which are associated with differentiation;
- maturation of the basement membrane occurs with the development of cell adhesion and expression of integrins a6 and b4;
- rapid deposition of dermal matrix takes place;
- migration of melanocytes begins from the neural crest;
- Langerhans' cells are detected originating in the fetal thymus and bone marrow; and
- Merkel cells are seen initially in the epidermis and subsequently in the dermis.

The early fetal period from 11 to 14 weeks sees the development of the hair follicles within the skin. The skin adnexal structures continue to develop and mature into the midfetal period at 15–20 weeks. As the fetus grows in the late period, 20–40 weeks, acceleration of stratum corneum can be identified in specific regions, including the palms, soles, face, and scalp. There is a close association between keratinization and hair follicle development, with the stratum corneum developing initially in the perifollicular regions. The mature stratum corneum is a structure that develops in the late fetal period: a combination of the terminally differentiated keratinocytes forming a cornified envelope and lipid extrusion from the abundant lamellar bodies of the granular layer keratinocytes.

Embryologically, the neural tissue and the epidermis are derived from ectoderm. By the end of the fourth week of embryonic development, the neural ectoderm has separated from the surface ectoderm, forming the neural tube. Under normal conditions, the nerve endings will never be exposed to the external environment. The skin forms the interface and has developed as the interactive responsive surface. In the following weeks, mesoblastic cells from the neural crest migrate into the skin as melanoblasts and the early nerve fibers develop as the vasculature migrates into mesodermal elements destined to become dermis. A close association is seen in development with the skin, developing as a tactile organ providing feedback information from the surface to the developing central nervous system (CNS). An understanding of neural plasticity of the CNS and the peripheral nerve field underpins the self-organization principle. The co-development is put forward as an explanation of the coevolution of the human CNS and the skin as an adaptive dynamic interface.

In the fully developed skin, there are cells from all three embryological layers in a complex framework of ECM. There are functions common to all skin areas, but there has also been adaptation to the specific functions of given body sites that are seen even at the early fetal stages.

The mature epidermis is composed primarily of keratinocytes arising from a layer of basal cells situated on the basement membrane. As the keratinocytes differentiate, they form a stratified squamous epithelium. As the cells undergo terminal differentiation, they lose their nuclei and form a highly cross-linked protein-based layer of keratin. The basal cells are in intimate contact with terminal dendrites: synapse-like structures have been described between nerve endings and keratinocytes. The melanocytes are situated in the basal layer with the melanosomes being

transferred to the differentiating keratinocytes giving the color of the skin owing to pigment load. The cells linking to the immune system, Langerhans' giant cells and dendritic cells, are also present in the epidermis. The epidermis is specialized to the body sites, most noticeably with the thickened cornified layer of the sole and palm [21].

The dermis is attached to the epidermis at the dermal epidermal junction by the basement membrane morphologically arranged as the rete pegs, which are exaggerated in the glabrous skin areas. The dermis is mainly connective tissue, predominantly collagen, with elastin seen in the superficial papillary dermis. The fibroblast is the cell that produces the ECM, which is specialized over differing body sites; areas such as the groin and axilla are thin and more elastic than the thicker, more rigid dermis of the back. Cells of hematopoietic origin such as lymphocytes and macrophages migrate into the dermis as the vascular bed becomes established and are involved in immune surveillance. The neural and vascular networks maintain the skin and facilitate the functions of the dynamic interactive skin interface [22].

The investigation of wound healing, in parallel with an understanding of skin development and functions, gives us the opportunity to develop innovative methods further to apply to TE. The skin has developed specifically in relation to the multiple functions it performs. As an active organ, it is responsive to changes in the external and internal environment, pivotal in maintaining the body's homeostasis. Our knowledge of skin functions is still growing and includes:

- a semipermeable barrier, overcoat, and suit of armor;
- thermoregulation and refrigerator;
- antibacterial and waterproof;
- UV protection and sunshade;
- sensory receptor that is sensitive to the touch of a feather, temperature, and pain;
- self-regenerating, withstanding the wear and tear of a lifetime;
- capable of rapid repair, executing its own running repairs;
- immune modulation;
- psychological interaction; and
- vitamin production.

The loss of skin integrity can result in severe morbidity and even mortality. The body needs a barrier against the atmosphere to maintain homeostasis. The production of the stratum corneum can be mimicked in tissue culture by exposing a sheet of keratinocytes in culture to an air–liquid interface [23], but it requires maturation in situ to develop the "smart material" of the enucleated cell bodies fully and the extracellular lipoproteins moisturized by vitamin E–producing sebum.

The keratinocytes produce surface proteins that are antimicrobial in the first line of defense against colonizing bacteria on the skin surface [24]. The expression of these proteins changes as the keratinocyte is stressed, as in wounding or culturing, such that protection from microbial invasion is highlighted.

The multiple and specific sensory inputs to the skin are pivotal in regulating the body's temperature and immune responses and psychological responses via neural and neuroendocrine control systems. Animal studies have highlighted the sensory role of the skin in normal development; touch is associated with growth potential of the internal solid organs [21].

The skin is also profoundly influenced by the pathophysiology of the individual, with cutaneous changes in anatomy and physiology being linked to many disease processes.

With this expanding body of knowledge with respect to the skin, we engage the fields of TE to provide solutions to repair and replace skin defects to maintain function and avoid scarring. To do so, we also need a working knowledge of the skin response to injury and tissue loss: the processes of wound healing [25].

Briefly, wound healing in skin is a complex series of cascading events that has been described in three overlapping stages from the initial inflammation to tissue formation and subsequent tissue remodeling [26].

The initial response is clot formation to achieve hemostasis. The activation of platelets releases the contents of their α-granules, resulting in activation of the clotting cascade and the release of adhesive proteins forming the matrix of the clot, e.g., fibrin and chemotactic factors and growth factors into the wounded area. The coagulation pathway links to activation of the complement pathway's facilitation of the recruitment of neutrophils needed to facilitate the inflammatory response by removing cellular debris and microorganisms.

The ingrowth of new vessels as granulation tissue is initiated and the keratinocytes at the wound edge mobilize to commence reepithelialization. Macrophages migrate to the wound, releasing multiple protein growth factors as the wound response progresses to the repair phase. Both hemopoietic and mesenchymal stem cells (MSCs) from the

circulation are attracted into the wound with fibroblasts producing ECM, some as myofibroblasts associated with wound contraction.

There is an increased interest in the interactions between the keratinocytes and underlying fibroblasts as the matrix is remodeled and the new basement membrane develops. As the keratinocyte is exposed to collagen, it secretes collagenase and as the basement membrane integrity is restored, the cells revert to their normal phenotype in the situation in which healing is achieved without scarring. Molecular cues after inflammation are associated with positional information and assist in driving the cell−ECM interactions.

In situations of more extensive tissue damage, the fibroblast transforms to a scar phenotype with the production of disordered collagen, resulting in an ECM framework that differs chemically, architecturally, and mechanically from the native skin construct. The interactions between the ECM and the fibroblasts respond to the changes as healing progresses from tissue repair to remodeling. The initial migratory fibroblast transitions to the profibrotic phenotype, producing ECM proteins. In the remodeling phase, the cell number within the dermis or scar reduces as the cells undergo apoptosis. The construct is dynamic and responsive to cellular, chemical, and mechanical cues.

Knowledge of the wound-healing progression over time allows the TE skin solution to be clinically integrated into the process and may be directed toward a number of target strategies: the control of cells in growth, the genetic manipulation of cells to express a given phenotype, seeding of the retained dermis with cells from the dermal epidermal junction, removal of the full thickness of the area of compromised skin, and replacement with a specifically tailored TE construct.

POTENTIAL PREREQUISITE REQUIREMENTS FOR TISSUE ENGINEERED SKIN SOLUTIONS

The skin matures from the softness of the newborn to the skin in old age with the loss of elasticity and reduced potential for repair [27]. We believe that the young heal rapidly but scar aggressively, in contrast to the elderly, who heal more slowly and scar less [28]. Regeneration of the skin without functional or aesthetic deficit, rather than enhanced repair, remains the ultimate goal of wound-healing therapies [29]. However, the degree of scarring and the quality of the repair depend highly on the time taken to heal, with faster healing correlating to improved outcome [8]. The availability of the TE skin for timely use is a key factor balancing the issues of allograft to autograft and biological to nonbiological solutions. The differences in wound-healing responses with age, the condition of the patient, and the etiology of the defect will have implications when harvesting donor tissue for TE and will potentially influence the choice of TE technique [30].

The skin surface is continually replaced under normal conditions and the morphology is retained over the years with changes seen as a result of injury, pathology, and aging. The capacity to regenerate and self-organize becomes overwhelmed in all but trivial injuries such that the repair forms a scar that all too often is debilitating, both physically and psychologically. The traditional approach to reducing the time to healing a skin defect has been to graft skin. Full-thickness skin grafts (FTSG) will give the best scar result but appropriate donor sites to match with recipient sites can lead to skin mismatch with retention of the donor site characteristics, as seen when an FTSG from the groin is used to release a contracture on the palm of the hand. The donor site availability for split-thickness skin grafting (SSG) may be limited in size when large body surface areas are compromised as in burn injury or giant nevi. The area of cover of a given donor site can be increased by meshing or expanding, as in the Meek technique [31]. The expansion of the SSG is associated with small areas of wound healing by secondary intention and a poorer scar outcome. It is the desire to eliminate or at least reduce the scar by reducing donor site morbidity that has driven TE of skin over the past several decades.

Essential factors to achieving healing are:

- a source of cell's capable of differentiating into the tissue and
- an ECM capable of supporting the cells.

With an increased understanding of the skin physiology and interactions with the internal and external environment, we need to also consider:

- the 3D spacial information of the area under repair and
- feedback from the surface to facilitate self-organization.

The ideal needs for a TE skin replacement continue to be debated and are related to the clinical indications for use. Variations in skin with body site and age dictate that a tailored solution is required. Furthermore, the mechanism of

skin loss has a profound impact on the local and systemic response; when comparing surgical excision with a burn injury, it is seen that the burn is associated with an extreme inflammatory response [32]. The interventions and timing of the interventions are important considerations when preparing the wound for the TE construct. In our group, in treating acute burn injury and scarring, we have been guided by the following basic requirements [33]:

- rapidly available
- autologous
- site-matched
- reliable wound adherence
- minimal donor site morbidity
- clinically manageable
- improved quality of scar
- affordable

The list could be debated and expanded according to clinical needs and opportunities such as the use of allograft MSCs with their demonstrated immune privilege [34]. However, the improvement of outcome by clinically manageable affordable solutions affords a solid benchmark.

For TE to be successful it is clear that an in-depth working knowledge of the biology of the tissue is essential. There are a number of cell types within the skin, each with specific and often interrelated functions, some established within the construct, and others transitory from the circulation; their origin and maintenance of clonal capacity remain elusive. It is fundamental to the success of TE to have an understanding of essential information about how the cells relate to the other cells and the ECM of the skin to develop into a mature tissue construct [35].

The ECM scaffold is integral to tissue integrity. We know that the physical shape, mechanical properties, and chemical composition of the environment of a cell influence its phenotype. The predominate dermal cell responsible for ECM production, the fibroblast, changes its phenotype after injury and repair, as demonstrated in the epigenetic modification. The stiffness of the ECM is linked to the fibroblast phenotype, and an understanding of the mechanobiology is an opportunity to guide appropriate tissue regeneration. With the complexity of multiple factors at play, hierarchical mathematics will be useful such that the field of bioinformatics may assist in refining design in the future [36].

In designing of the TE solution, an understanding of this relationship will lead to increased clinical success guiding phenotypic expression. From an engineering perspective, there are technologies that will facilitate innovative clinical solutions such as the advanced modeling and fabrication for scaffold manufacture. Bioprinting has demonstrated the capacity to use the advancing knowledge of biomaterials, natural and synthetic, combined with the cells to produce scaffold cell constructs of increasing complexity. The materials for use need to [37]:

- be biocompatible;
- have properties compatible with mechanical aspects of bioprinting, such as extrusion;
- facilitate appropriate tissue-specific cell viability and function;
- maintain structural integrity and mechanical properties after printing;
- be responsive to chemical and cellular signaling; and
- integrate into the host tissue environment with vascular and neural connectivity.

The development of bioreactors to maintain viability and expand cell numbers associated with scaling up in an appropriately regulated laboratory is essential for cell culture–based techniques [23]. With the advent of bioprinting, it is essential to consider cell capabilities that ideally [37]:

- have the capacity for cell proliferation generating adequate cell numbers in a timely fashion, depending on the pathology being treated;
- are nonimmunogenic or autologous;
- are multipotential;
- are responsive to the environment; and
- have a capacity to integrate without fibrosis.

The increased use of MSCs as immune-privileged cells in a range of pathologies has led to the exploration of them as a source of cells in the TE field meeting these criteria [34]. The increasingly reliable generation of patient-specific induced pluripotential stem cells (iPSCs) provides an alternative source of cells with the capacity to be plastic down a range of lineages, affording potential for future differentiation into specialized cells of the skin adnexal structures [38]. As a source of cells for genetic engineering, treatment of cutaneous pathology becomes a possibility. Cells from

a range of sources can be guided by the introduction of signaling molecules to facilitate activity such as migration, proliferation, and differentiation, bringing us back to having an in-depth working knowledge and even expanding that knowledge base from embryology to wound healing to introduce the appropriate triggers. The cells can also be manipulated by physical changes such as hypoxia to stimulate responses or be guided by introducing magnetic nanoparticles to allow manipulation by external forces such as a magnetic field [39]. Putting the ECM and cells into proximity of a 3D structure is the beginning of the process that needs to be guided either in the laboratory or in situ in the wound to develop into the tissue construct.

It is clear from the experiences of the past several decades that many of the proposed TE skin solutions are disruptive technologies. It is essential that the TE skin not be designed for only a clinical problem and that it should be reproducible and reliable, but also that it should be linked to an education and training program so that its full clinical potential is realized [19].

CURRENT TISSUE ENGINEERING SKIN TECHNOLOGIES

Engineering principles have been applied to skin for many years with the development of medical devices to harvest SSG accurately and mesh the skin to allow expansion. The practice of tissue expansion is a well-established surgical tool for the development of skin by subcutaneous insertion of inflatable devices in vivo, which can be serially enlarged with the resulting development of the skin as demonstrated by cell proliferation [40]. The tissue-expanded skin has all layers and the complete characteristics of the donor site, including retention of adnexal structures and functional innervation [41].

There is increasing interest in the concept of tissue expansion in vitro with a full-thickness skin biopsy put under tension in a bioreactor system to maintain its viability and facilitate cell replication resulting in tissue growth [42]. However, these solutions are limited by time, area, and in some cases, donor site and scar outcome. The need remains to provide both rapid large surface area cover and complex site-specific specialized skin repair, with the ultimate goal of healing by regeneration, not scar repair.

The initial approach to TE skin was to separate the layers and consider the epidermis and dermis to be separate problems.

Work by Green in 1970 focused on the culture of keratinocytes into cell sheets suitable for grafting [43]. The solution to large surface area skin repair was to harvest cells from an uninjured donor site and to undertake laboratory-based tissue expansion. The resulting sheets of cells could be used to close the wound as would a traditional SSG. However, there were problems with the time taken to culture in the laboratory, fragility, adherence to the wound surface, and durability over time, because only the epidermis was replaced; in addition, cost was problematic [44]. In trying to solve some of these problems, there has been a development in the area of subconfluent cell transfer on a number of cell culture surfaces [45] in addition to the delivery of cells in suspension as an aerosol [46]. Subconfluent cells have more reliable adherence and are available in a shorter time frame, from 3 weeks for sheets to 5 days for subconfluent cultures [47]. The process of harvesting cells from the dermal epidermal junction by enzymatic and physical dissociation has been used for immediate delivery of a noncultured cell population to the wound [48]. The cells are a mixed cell population in the same ratio as that seen in the normal skin construct, because there has been no selection of cell populations seen when culturing. The maintenance of the melanocytes enables the development of appropriate pigmentation [49]. The cells adhere, migrate, and proliferate across the wound surface and then differentiate and self-organize into a mature epidermis. The scar outcome is intimately linked to the underlying wound bed, which will be discussed in a later section.

The development of a suitable dermal scaffold is also the focus of the TE field [50]. Topographical features are known to influence cell behavior through a phenomenon known as “contact guidance,” and alteration in the size of the surface detail can elicit different cell responses [51]. Running parallel to studies on the epidermal cell culture was work by Yannas and Burke on dermal replacement, which culminated in the first commercially available product, Integra [52]. The concept of tissue-guided regeneration within an architectural framework is in clinical practice [53]. Underpinning research on the composition and construction demonstrated the importance of considering both aspects: a combination of bovine collagen coated with glycosaminoglycan but with a pore size of less than 60 mm or greater than 100 mm, resulting in disordered granulation tissue, with the optimal pore size resulting in the migrating cells expressing a reticular dermal fibroblast phenotype. The main drawback with Integra is that it addresses only the dermal aspect, with the outer layer on silicone acting as a pseud-epidermis for the period of vascularization, usually 3 weeks before a second surgical procedure is needed to repair the epidermis [54]. The epidermal repair is with a thin SSG, which may be meshed to cover a larger area than the donor site with epidermal cells to speed the time to

healing and reduce the mesh scar pattern [55]. The two-stage problem has been addressed by trying to reduce the time to vascularization or by developing constructs that can be used with an SSG at the same procedure. Apligraft, Matriderm, and Pelnec are dermal templates marketed to provide appropriate topography and matrix properties to promote cell migration into the wound, improve healing, and reduce scarring as a one-stage procedure with SSG [56]. An alternative is to use our knowledge of healing as we see cells migrating from areas of the skin adnexal structures in the dermis to form the new epidermal layer. Introduced cells harvested from the dermal epidermal junction seeded into Integra will migrate and organize into a new epidermis with an established dermal epidermal junction within 3 weeks [57]. The use of Integra is a clear demonstration of the potential of tissue-guided regeneration with the expression of a cell phenotype guided by the morphology and chemistry of the matrix [58].

It is well-established that cells change their phenotype in response to changes in their environment [59]. The development of suitable technologies to generate an optimal environment for wound healing is important to enhancing cell response to tissue injury, reducing the time to heal and improving the outcome. Our knowledge of ECM−cell interactions is increasing with the recognition of the cell signaling by nanoscale structures on cell surfaces with roles in attachment and cell migration [60]. Developments in nanotechnology have opened up possibilities in TE to improve scaffold design, but relatively little is known about how changes in topography at the nanoscale affect cell behavior [61]. The scaffolds can be manufactured to address specific skin functions: to protect against injury from loss of fluid and proteins, enable the removal of exudates, inhibit exogenous microorganism invasion, and improve the aesthetic appearance of the wound site [62]. Scaffolds are generally matrices of synthetic and/or natural polymers fabricated by various techniques including solvent-casting, gas foaming, electrospinning, phase separation, freeze-drying, melt molding, and solid free-form fabrication [63]. Critical to their performance is reproducibility, with control over pore size and the distribution of pores, removal of residual toxic organic solvents, and the control of the inflammatory and immune responses owing to polymer degradation and the associated by-products [64]. The printing technology could conceivably be used directly in wound bed preparation, providing initial protection and a blueprint for cell migration and guided tissue regeneration [65].

Our group has explored anodic aluminium oxide (AAO) as a potential scaffold or template in TE [66]. Self-organized oxide growth under controlled conditions generates a densely packed hexagonal array of uniform-size nanopores aligned perpendicular to the surface of the AAO film. The size of the pores can be nanoengineered by manipulating the anodization time and voltage, the anodizing electrolyte, and/or the time of postchemical etching. Aluminum oxide is well-known for its biocompatibility in the human body; it is inert, stable, and nonreactive, which makes it suitable for TE applications. Engineering of surfaces to manipulate healing is a rapidly expanding area; interactive dressing systems is in widespread clinical use. With the realization of the impact of surface topography and chemistry on cell expression and developing nanoengineering techniques such as electrospinning and electrospraying, there is increasing interest in smart surface technology skin healing [67]. Biocompatible polymeric self-assembling nanofiber constructs have the advantage of a large surface area that can be linked to bioactive compounds. The release of the bioactive compounds can be controlled by intrinsic factors such as in a hydrogel, release kinetics, or extrinsic release triggers [67]. Exciting advances have been made in the area of nanocubes, nanocages, and nanorods as primary candidates to be studied for the phototherapeutic release of bioactive agents [68]. Clinically, the results of single cytokine applications have been disappointing and study of natural healing processes is a result of complex cascade interactions over time [68]. It is unsurprising that we cannot achieve with a single cytokine administration what is the result of complex cell−ECM interactions. The aim of advancing technology is to mimic the structure and function of the ECM with the ability to adapt over time to the changing environment of the healing wound [69].

TE technologies are also used to test the potential impact of agents on the skin, such as in the cosmetic industry. The degree of complexity of laboratory-based testing systems vary from 3D-printed constructs at an air−liquid interface to produce a differentiated epidermal layer to techniques such as the “scar in a jar” system for testing biological activity of drugs targeting specific pathways in fibrosis [70].

Bringing together the scaffold and cellular components has been successful with the development of multilayered constructs seeded with multiple cell types [71]. Clinical series have been presented demonstrating a soft supple skin but with the persistent problem of poor color match [72]. An understanding of the interdependence of the cells of the dermal epidermal junction may well be important to the development of skin constructs with the appropriate melanocyte function [73]. The main drawback in the clinical use of complex laboratory-based constructs is the time taken in the laboratory [74]. However, the potential to use such a technology in timed reconstructive surgery as opposed to acute trauma is beneficial with the ability to tailor the skin construct to the planned defect. The 3D distribution of cells within the wound has been addressed by the innovative use of “inkjet printing” technology, with the cells “printed,” controlled by the shape and depth of the defect [75]. The cell type within the system can be

changed with the fibroblasts being laid down before the keratinocytes [76]. ECM and other proteins can also be introduced into the system, such as hair-based keratins, chemical-processing substrates from biological origins to develop innovative scaffold solutions to enhance cell performance within the constructs.

The source of the cells and the guidance along a path of differentiation are critical elements for a functional skin construct. The use of iPSCs offers the potential for differentiation into multiple cell types, including fibroblasts, keratinocytes, and melanocytes [77]. Guidance of cell differentiation and ECM production may hold the answer to replicating the adnexal structures. The iPSCs also have potential in gene-based therapies in treating skin disorders such as epidermolysis bullosa [78]. The use of the cells as therapeutic delivery agents may broaden therapeutic opportunities.

The time to availability of a TE technique is a key driver to their clinical employment. The use of allograft materials allows an "off-the-shelf" approach, whereas autograft material may take time in the expansion phase [79]. The use of scaffolds alone can provide an advanced wound management system to facilitate healing and can replace a tissue defect guiding repair as it is replaced or provide a permanent solution. The use of cells alone can also provide a surface epithelium that can modulate the underlying healing, form a mosaic of cells of intrinsic and extrinsic origin guiding repair, or provide a permanent surface [80].

The combination of the two elements can provide an advanced skin repair solution, but because the differentiation of the construct is more advanced, the time taken in the processes is prolonged. Consideration of the time taken has led to an investigation of the construct being used in the immature form differentiating in situ. It is clear that TE provides a range of innovative solutions that are useful for many wound and skin replacement areas. Effective use of the technology hinges on the clinician understanding the wound preparation and the aim of the repair. The wound assessment drives the initial clinical decisions directing management in terms of resuscitation, tissue salvage, and infection control, and then planning the repair. An understanding of the range of TE solutions is essential to appropriate clinical implementation.

Consideration of the hypodermis in the resulting functional unit adds a further degree of complexity and opportunity to advance the quality of the tissue construct [81]. Fat grafting to improve the contour of the hypodermis has been shown to be associated with modulation of the cutaneous scarring. The cellular mechanism of the interaction is of interest when considering the opportunity for systemic, regional, or local solutions for a given clinical problem.

TISSUE ENGINEERING SKIN SOLUTIONS IN CLINICAL PRACTICE

The following clinical case of an extensive 65% total body surface area burn injury associated with multiple fractures and pneumothorax is used to demonstrate the decision making and options available in current practice. The initial stabilization and resuscitation is life-saving, along with attention to infection control using Acticoat, a nanocrystalline silver dressing. Once stable, surgical debridement is planned to excise the areas of skin that cannot be salvaged. Although the timing of debridement is vigorously debated with respect to controlling ongoing inflammation and improving outcome potential, an important element in decision making is what is available to cover the debrided wounds, either as a temporary measure or as a permanent replacement. A full-thickness wound requires dermal and epidermal repair for the optimal outcome. In large−surface area wounds, standard SSG is not possible in one procedure owing to restricted donor sites; the SSG can be meshed to achieve healing in a larger area but healing of the interstices by secondary intention often leaves an unsightly meshed pattern scar. Cadaver allograft has been widely used but provides only a temporary solution and will require serial cover as the donor sites heal. A combination of allograft dermis and sheet cultured epithelial autograft (CEA) has been reported to result in a composite repair with retention of the dermal elements. The CEA sheets may take 21 days to culture, or more timely availability can be achieved using preconfluent cultures on carriers or delivered as suspension. The dermis can be replaced with a number of off-the-shelf products such as Matriderm, Pelnec, or Apligraft. The TE technique most widely reported to date remains Integra. The use of composite TE skin could be considered to augment the second stage of the repair with Integra, because it takes time in laboratory preparation.

In this case, Integra was used to reconstruct the dermis in the areas of full-thickness excision, as seen in Fig. 73.1, in which the Integra is held in place with a combination of staples and dressings to facilitate vascularization and integration of the construct. In areas where dermis could be salvaged, cells were harvested from the dermal epidermal junction of uninjured skin using a ReCell kit with Biobrane as a dressing. The main drawback with Integra is the period of vascularization of 3 weeks before proceeding to repair of the epidermis. The epidermis was repaired using a thin meshed SSG 1−3 combined with a noncultured cell suspension from the ReCell kit to reduce the meshed pattern scar. The healing is seen in Fig. 73.2 with the mash pattern fading well in the upper compared with the lower

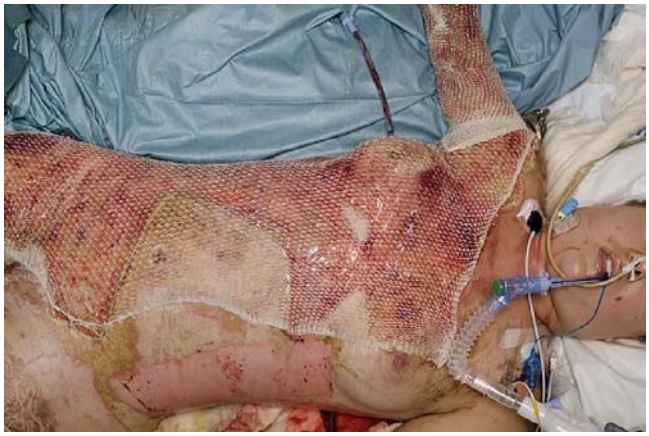

FIGURE 73.1 Postsurgical debridement of full-thickness burn over 65% of the total body surface area (TBSA) and replacement with Integra dermal scaffold held in place with elastic netting; area of a partial-thickness burn on the abdomen treated with autologous cells under a protective dressing; and uninjured skin donor site on the left flank.

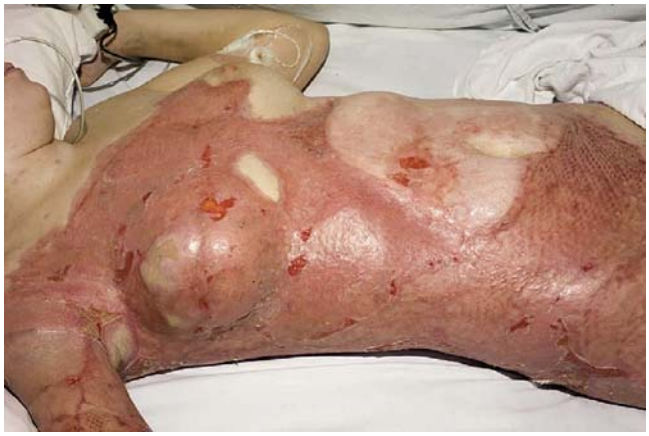

FIGURE 73.2 Post-second procedure to repair the epidermal layer using a combination of meshed split-thickness graft with a noncultured autologous cell spray.

abdomen. At the time of scar maturity in Fig. 73.3, the scar situation demonstrates the issues faced by the patients [82]:

- contour deflect caused by the removed subcutaneous fat layer,
- persistent mesh pattern in the lower scar,
- mismatch of pigmentation,
- contracture bands distorting the anatomy, and
- the repair resulting in a scar.

There has clearly been progress; the development of TE techniques is intimately linked to the advancing survival and quality of scar in patients with major burn injuries. However, there is a clear need to continue to develop TE with the aim of total 3D soft tissue and skin replacement [83].

THE FUTURE

The vision of scarless healing has led to the exploration of regeneration and the interplay among genes, cells, and tissues [84]. Pluripotential stem cells are present within each individual; the drivers of the cells down a regenerative path are an as yet unknown but exciting area of research with promise for the future [85]. The introduction of

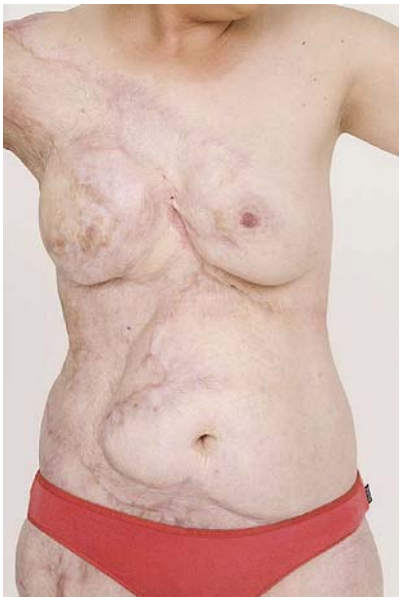

FIGURE 73.3 Two years after injury, before planned reconstructive surgery.

allograft stem cells such as MSCs may provide an alternative source of cell regeneration [86]. Understanding that every intervention from the time of injury influences the scar worn for life has pushed research in a multitude of directions. TE of skin is an exciting area that already has made a significant clinical impact [87]. However, we are far from the routine provision of technologies and strategies to provide site-matched, fully functional skin [88]. Great progress has been made in the areas of dermal templates and cell-based therapies and bringing the two elements together in skin constructs. The clinical implementation of TE skin solutions, along with the use of interactive surface dressing systems, has improved outcomes.

The task is far from completed:

- Skin adnexal structures are elusive [89].
- Timely availability of TE skin remains problematic [3].

In addition, more work is needed in:

- harnessing the explosion in smart material technology [90];
- developing vascular constructs, the small vessel problem [91];
- understanding the drivers of tissue-guided regeneration [92];
- understanding the concept of self-organization and the bioinformatics behind morphology [93];
- investigating the impact of both reinnervation and neural plasticity and its role in scarless healing [94];
- understanding the barriers to clinical translation [95]; and
- developing regulatory pathways for novel solutions to ensure safe but timely availability [96].

At the high-tech end of the spectrum, we may consider bringing together laser surface imaging linked to fabrication to build bioreactors with the shape of the defect, with the "smart" cytokine-loaded scaffold materials tailored to the correct 3D shape. Cells of the appropriate body site could then be introduced into the scaffold by cell-printing methods in the individualized bioreactor and the flow of tissue culture medium used to induce small-vessel formation. At the time of transplant, the application of external techniques such as infrared could control the release of biologically active molecules from the "smart" scaffold surface to ensure, for instance, reinnervation and restoration of function with the capacity to integrate into the body. Understanding of linking local tissue replacement with systemic integration is essential to facilitate the linking partially cellular constructs with a 3D framework architecture facilitating secondary cell migration and ingrowth. An alternative to the individualized bioreactor could be the wound itself, prepared by "smart" surfaces and directly seeded with cells. With the understanding of the drivers to self-organization, they could be used to enhance in situ tissue-guided regeneration.

CONCLUSION

Over the past several decades, the concept of TE has been an area of active research, investigating innovative solutions. Multiple combinations of 3D engineered scaffolds exist with a functional cell load to produce tissue over time, which is the fourth dimension of skin repair fundamental to the clinical selection of technique.

The elements required for tissue repair are:

- a source of cells capable of differentiation into the lost tissue,
- an architectural framework for cells to migrate into and express the appropriate phenotype,
- 3D spatial information of the damaged tissue and the relationship to the surrounding viable uninjured tissue interface, and
- a feedback mechanism to guide self-organization.

In Jul. 2005, the *Medical Journal of Australia* published a vision of clinical care in a number of disciplines in 50 years' time:

> Assessment is key in understanding the extent of injury. Debridement is focused on tissue salvage.
>
> Reconstruction balances repair with regeneration. Investigation of multimodality, multiscale characterisation, including confocal microscopy and synchrotron technology will quantify assessment.
>
> Debridement using autolytic inflammatory control techniques with image guided physical methods will ensure the vital tissue frameworks are retained. Tissue guided regeneration afforded by self-assembly nano-particles will provide the framework to guide cells to express the appropriate phenotype in reconstruction.
>
> To solve the clinical problem a multi-disciplinary scientific approach is needed to ensure the quality of the scar is worth the pain of survival.

Many of the technologies highlighted are available, but the significant need for clinical translation remains to move along the innovation pathway to ensure safe implementation into health care systems. Progress requires collaboration at all stages from basic science to clinical trial design and health economics, driven by improved clinical outcomes. Translation of new technologies into health systems requires the rigor of a research framework to identify and measure the impact of innovation in communication and education. Close working relationships between basic research and clinical service delivery are essential. Furthermore, the scientific and clinical advances need to be in line with regulation programs and linked to commercial interest to ensure their widespread availability.

If the solution to the problem is scarless healing by a regenerative repair process and the aim is to improve the outcome from injury by restoring function, the future must blend the long-term vision with incremental short-term improvements.

There has been great progress in TE of skin, and it is an exciting area that offers tangible clinical solutions with an enormous potential for further improvement. The range of potential solutions that have developed is clear recognition of the complexity of the problem and the unique requirements relating to body site, patient, and pathology and the extent of the skin needing to be replaced, repaired, or regenerated. The challenge we face is to capitalize on that tradition and link to the opportunities afforded by unprecedented growth in science and technology, to ensure the quality of the scar outcome is worth the pain of survival.

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CHAPTER

74

Regenerative Medicine Approaches for Engineering a Human Hair Follicle

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INTRODUCTION

The hair shaft consists of terminally differentiated keratinocytes, which are tightly compacted to form the hair shaft. Hair shafts are made by the hair follicle, a complex miniorgan that is formed primarily during fetal and perinatal skin development [1–3]. Hair follicle formation involves tightly coordinated interactions between the ectoderm and mesoderm with ectodermal stem cells giving rise to all epithelial components of mesoderm cells forming the follicular dermal papilla, as well as the connective tissue sheath [4]. It is composed of an outer root sheath (ORS), inner root sheath, and hair shaft. Under normal homeostasis the hair follicle undergoes a cycling, which is characterized by the formation of a new hair and shedding of an old hair through the growth cycle (anagen), apoptotic regression (catagen), and a resting phase (telogen) (Fig. 74.1). A very unique characteristic of the mammalian hair follicle is its lifelong recapitulation of its molecular embryogenesis each time it enters into anagen. Dermal papilla cells (DPs) are located at the base of the follicle and are surrounded by epithelial matrix cells. DPs support the proliferation as well as the differentiation of epithelial matrix cells and activate bulge stem cells during the transition from telogen to anagen. The bulge is part of the ORS that is close to the sebaceous gland and the interfollicular epidermis. Bulge cells give rise to all of the epithelial cell types during anagen, as well as the sebaceous gland and overlying epidermis. In anagen, cells at the base of the hair follicle proliferate and migrate up the follicle to form a new hair. Simultaneously, the bulge cells give rise to progeny. Robust activity during anagen requires an increase in nutrients and the phase is therefore also associated with new blood vessel formation to facilitate a more rapid supply of these nutrients.

Much has been learned about the regeneration of skin and skin appendages through wound-healing studies and cell- and tissue-based treatments. Some of the earliest approved products in regenerative medicine were cell-based three-dimensional engineered tissues for the treatment of acute and chronic wounds [5]. These products taught us the importance of naturally secreted growth factors and matrix proteins in inducing healthy granulation tissue, angiogenesis, and reepithelialization. Unfortunately, none of the products induced the formation of critical skin appendages, including hair follicles and sweat glands. Anecdotal evidence in wound care reported cases with badly debrided skin healed with the formation of new hairs. [5a] It had been previously thought that mammalian hair follicles only form during fetal development [6] and that the loss of hair after birth was permanent. Reports that hair follicles could be developed de novo after wounding were published decades ago in mice [7], rabbits [8], and humans [9] but these were not accepted due to the lack of evidence of follicular neogenesis. In 2007 a pivotal paper was published showing that hair follicles formed de novo after wounding in genetically normal adult mice [10]. Evidence was presented that nascent follicles arose from epithelial cells outside of the stem cell niche and suggested that wound epidermal cells can assume a hair follicle stem cell phenotype. Overexpression of the Wnt ligand in the epidermis increased the number of new follicles, while inhibition of Wnt signaling completely stopped the folliculogenesis. The regenerated follicles produced new hairs and cycled up to three times during the 90 days following wounding, supporting the presence of functional stem cells in the newly formed follicle. The de novo formation of hair follicles in the adult mammal mimics embryogenesis at the molecular level and raises the possibility

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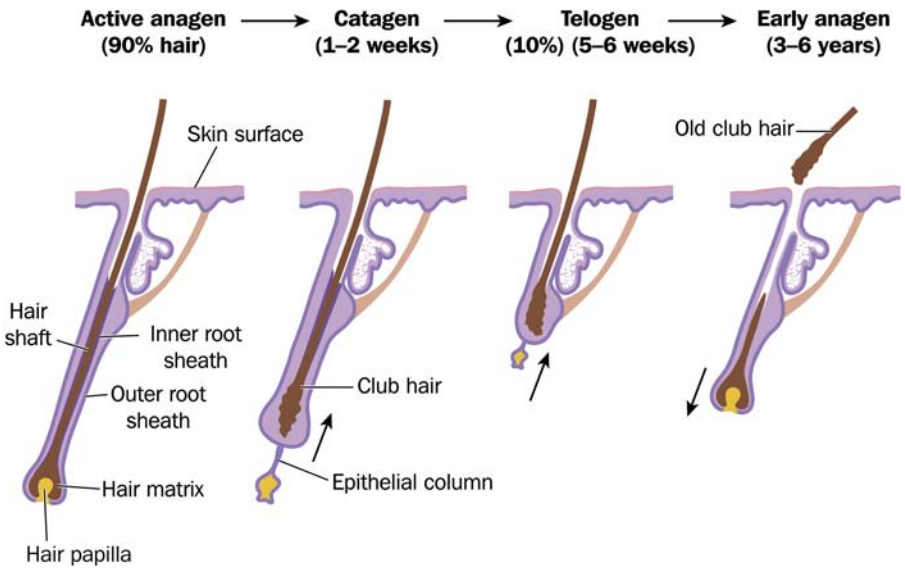


FIGURE 74.1 The normal hair follicle cycle.

of forming new follicles from preexisting hair follicle stem cells in the scalp or from controlled wounding when used in combination with Wnts and other cell stimulatory molecules. A number of preclinical and clinical studies have been performed using cells and cell- and tissue-derived materials to induce stem cell proliferation in the dying follicle or create new follicles in the scalp.

The onset of hair loss is characterized by a much shorter than normal anagen (growth phase) and a reduction in hair density [4]. Given this mechanism, the development of a successful hair loss treatment needs to address two key processes: the transformation of vellus hairs into terminal hairs by stimulating and extending the length of the anagen phase, and the induction and maintenance of the anagen across the entire area effected by miniaturized follicles that result in increased hair density. The number of patients with alopecia has been on the rise and has been attributable to hormones and genetic factors [11], as well as autoimmune diseases, chemical and heat stress, medicines, and psychological stresses [12,13]. Androgenetic alopecia (AGA) is the most common form of hair loss in men during which terminal hairs turn into vellus hairs under the influence of dihydrotestosterone (DHT). Hair follicular miniaturization occurs as a consequence of progressively shorter anagen phases. To date the treatments of alopecia have focused on either blocking the DHT or inducing greater blood flow to help support anagen. These treatments need to be used daily, and within 2 weeks of stopping treatment there is a mass shedding of hair. What is needed is a treatment that will reactivate the miniaturized follicle and cause normal and sustained anagen. Research suggests that dormant hair follicles in human scalp affected by alopecia have a largely intact bulge stem cell population and a significantly reduced population of hair germ progenitors [14]. The hair germ progenitors become activated at the very onset of anagen, while the bulge stem cells become activated shortly after the actual initiation of anagen [15]. It is therefore conceivable that the reactivation of anagen in alopecia-affected telogen (resting) hair follicles may occur by the delivery of threshold levels of growth factors to induce bulge cells as opposed to triggering hair germ cells. The use of such a growth factor therapy has been studied through a variety of approaches, including autologous platelet-derived growth factors (PDGFs), growth factors from adipose cells grown under hypoxic conditions, growth factors from hypoxia-induced multipotent stem cells (HIMSCs), and the use of autologous stem cells to act as growth factor delivery units.

USE OF AUTOLOGOUS GROWTH FACTORS IN HAIR FOLLICLE REGENERATION

Autologous growth factors have been associated with the promotion of tissue regeneration in a number of medical applications. Early work with platelet-rich plasma (PRP) showed excellent generation of granulation tissue and subsequent reepithelialization of hard-to-heal diabetic ulcers. Autologous PRP is derived from centrifuging whole blood to collect a pellet of platelets. Alpha granules in activated platelets release numerous growth factors, including epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF-BB), transforming growth factor beta 1 (TGF-B1), and vascular endothelial growth factor (VEGF). Although initial studies

with PRP focused on the treatment of diabetic ulcers, PRP is routinely injected into the skin for wrinkle indications, it is injected into joints to reduce pain and inflammation, and some findings suggest that PRP may also significantly support hair follicle restoration. One such study involved the treatment of 19AGA patients with plasma rich in growth factors (PRGFs) [16]. Five PRGF injections were administered into the mid-dermal region and phototrichograms were performed at baseline and at 1 year following the last set of injections to assess follicle density/diameter and the terminal/vellus hair ratio. After PRGF therapy, mean hair density/diameter increased as did the terminal/vellus hair ratio. An improvement was also noted in epidermal thickness, perifollicular neoangiogenesis, terminal/miniaturized hair ratio, and cell proliferation. Although this was an uncontrolled clinical trial, the data do support a potential therapeutic effect of the autologous growth factors on hair follicle regeneration. Currently, a number of practitioners are looking at factors that can affect the ability of PRGFs to induce terminal hairs, including the age of the patient, vitamin D deficiencies, and general health.

USE OF ADIPOSE-DERIVED STEM CELLS AND THEIR CONDITIONED MEDIUM FOR HAIR GROWTH

Adipose-derived stem cells (ADSCs) are mesenchymal stem cells (MSCs) found within the stromal−vascular fraction of subcutaneous adipose tissue [17]. ADSCs self-renew, display a multilineage developmental plasticity, and have been used in a variety of tissue repair and regeneration clinical studies. It has been reported that ADSC transplantation promotes hair growth in animal experiments [18]. This is not surprising since hair regeneration depends on cell−cell interactions, as well as external signals surrounding the follicle. Communication between adipose tissue and the hair follicles is key and adipose cells play an important role in the progression of the hair cycle [19]. Canine ADSCs were isolated and cell differentiation into dermal papilla-like tissues (DPLTs) was induced by culturing them in a dermal papilla-forming medium. The trichogenicity (hair forming potential) of the differentiated cells (DPLTs) was assessed and athymic mice were treated either with control or the differentiated cells. DPLTs were seen to have a compact aggregated structure and they secreted a complex extracellular matrix component, as well as versican and alkaline phosphatase, two dermal papilla-specific proteins. New hair fibers were seen 15−20 days postinjection, with the greatest number of hairs seen in the treatment group. Regenerated hairs in the control group were predominantly found in the periphery of the wound and were characteristically short and thin. By contrast, the DPLTs-treated mice had new hairs throughout the treatment area, as well as a significantly higher amount of sebaceous glands at days 10 and 20 and increased vascularization. These data suggest that engineered canine DPLTs demonstrate characteristics of dermal papillae and have a positive effect on hair regeneration. A phase 1/2 trial is currently under way in the United States to test the ability of autologous ADSCs to promote hair growth in men with male pattern baldness.

A critical function of ADSCs is the secretion of growth factors that activate neighboring cells and mediate diverse skin-regenerative effects, including wound healing, antioxidant protection, and antiwrinkling [20]. ADSCs also secrete regenerative growth factors that participate in hair morphogenesis and regeneration. The hair growth promoting effect of ADSC-conditioned media (ADSC-CM) was studied by subcutaneous injection of the CM into C3H/NeH mice [21]. The CM contains a variety of growth factors, including VEGF, hepatocyte growth factor (HGF), insulin-like growth factor (IGF), and PDGF. The researchers discovered that culturing ADSCs under hypoxic conditions significantly improved their proliferative and self-renewal capacity and enhanced their growth factor secretion. The hypoxia-derived CM (HCM) induced the enhanced proliferation of human keratinocytes, as well as human follicle dermal papilla cells in vitro as compared to the CM from ADSCs grown under normoxic conditions (NCM). Based on these data, the induction of hair growth in 7-week-old C3H/HeN female mice ($n = 21$) treated with HCM was assessed. Mice received three subcutaneous injections of 100 μL NCM or HCM. Injections were given at days 0, 3, and 6 and skin darkening as an early indicator for hair follicle formation was monitored for a 12-week period of time. After 8 weeks, dark spots and hair regeneration were seen in the areas of injection with complete hair growth seen by 12 weeks in the HCM-treated animals. Dark spots and hair regrowth were not seen in the NCM-treated mice. Clinical pilot studies have been performed to study the hair growth effect of ADSC-CM on both male and female patients [22]. In women with female pattern hair loss, ADSC-CM was applied to the scalp after microneedling, the use of ultrafine needles to interrupt the skin barrier. After 12 weeks there was a significant increase in both hair thickness and hair density, and no adverse reactions were reported. The researchers also studied the effect of intradermal injections in both men and women with hair loss. Twenty-two patients received six sets of injections every 3−5 weeks and this treatment regimen resulted in an increase in hair numbers after week 12. No large, controlled, double-blinded clinical studies have been conducted to date and research is being done to enhance the hair regeneration potential of ADSC-CM.

TISSUE-DERIVED MATERIALS FOR HAIR REGENERATION

Human placental extract (*Hominis placenta* [HP]) has been used for decades in China and Korea as a therapeutic agent for liver regeneration and endocrine abnormalities. HP extract has been shown to have antiinflammatory, wound-healing, and antioxidant effects in the clinical setting [23]. Initial studies with cow placenta showed an increase in hair density in the mouse model [24] and this led to an investigation into whether HP treatment could influence hair growth in mice. Fifteen 7-week-old male C57BL/6 mice were divided into control, minoxidil, or HP-treated groups. Animals were depilated to synchronize hair follicle growth to the anagen phase. Animals received topical treatment of the controls or treatment once daily for 15 days, after which time measurements were taken for hair growth and thickness by dermoscopy. Immunohistochemistry was also performed on skin sections from all animals to assess BrdU incorporation and DNA synthesis. Results demonstrated an increase in hair number, hair shaft diameter, and density in the HP-treated animals as compared to the control animals. In addition, high levels of BrdU incorporation were observed in the dermal papilla, ORS, and connective tissue of the HP-treated animals. Proliferating cell nuclear antigen was highly expressed in the lower regions of the hair matrix, indicating induced DNA synthesis in the treatment group. Fibroblast growth factor 7 (FGF-7), a critical growth factor in the stimulation of anagen, was seen to have an increased expression in the outer layer of the hair shaft and surface epithelium in the HP-treated group. Placenta extracts have been utilized in a variety of clinical indications, including wound healing where the growth factors, amino acids, and angiogenic-promoting agents have proven to be beneficial. Hair follicle cycling has a great deal in common with the processes involved in wound healing and HP continues to be assessed as an anagen-promoting stimulus.

ADDITIONAL STUDIES ON SECRETED GROWTH FACTORS AND HAIR GROWTH

Additional preclinical studies have been performed to build on the research showing the importance of Wnt proteins and other growth factors in the stimulation of follicular stem cells to induce sustainable anagen. In one such study, MSCs were engineered to overexpress Wnt1a [25]. Wnt-CM was injected into the murine model discussed earlier and resulted in accelerated hair follicle progression from telogen to anagen and enhanced alkaline phosphatase expression in the DP region. Of great significance is the fact that hair induction-related genes were upregulated even when they had been first selectively downregulated by treatment with DHT. This study clearly demonstrated that MSC-produced Wnt1a promoted the ability of the DP cells to induce hair cycling and regeneration in a physiological manner in a model simulating male pattern baldness. In another study, neural stem cell (NSC) extract was used to stimulate the hair follicle niche to induce hair growth in the mouse model. Expression levels of multiple growth factors and signaling factors were studied and it was reported that NSC extract enhanced hair growth by activating hair follicle niches via the coregulation of TGF-B and bone morphogenetic protein (BMP) signaling pathways in the telogen phase. The activation and differentiation of intrafollicular hair follicle stem cells, extrafollicular DPCs, and matrix cells was also seen both in vitro and in vivo. Key growth factors, including HGF, IGF-1, keratinocyte growth factor (KGF), and VEGF, were also increased to enhance hair growth stimulation. This study clearly demonstrated the ability of NSC extract to promote hair growth through the direct regulation of hair follicle niches via the TGF-B and BMP signaling pathways, as well as the induction of critical growth factors [26].

SIMULATING THE EMBRYONIC ENVIRONMENT

In the mid-1990s it was reported that scarless healing occurs in the embryonic/fetal environment even after extensive surgery [27]. The embryonic environment is known to be characterized by rapid cell growth and cell differentiation, and embryonic tissue develops under low oxygen (hypoxic) conditions, and, unlike adult tissue, it contains predominantly collagens III and V and low levels of TGF-B and bFGF. Ezashi et al. [28] have shown that mimicking the embryonic condition of hypoxia enhances the growth of human embryonic stem cells (hESCs) and both hESCs and adult stem cells show improved maintenance of their undifferentiated state under low oxygen conditions [28–30]. To assess the effect of embryonic conditions on normal cells, neonatal fibroblasts were seeded onto dextran beads and grown in suspension in controlled closed bioreactors under 5% oxygen. Under these cell culture conditions, over 5000 genes were differentially expressed as compared to identical growth conditions with normal oxygen. A large number of the upregulated genes were associated with multipotent stem cells, including SOX 2, Oct4,

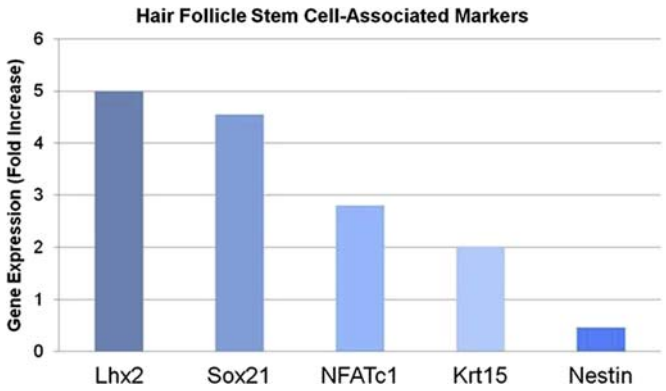


FIGURE 74.2 Expression of hair follicle-related stem cell markers in neonatal cells grown under simulated embryonic conditions.

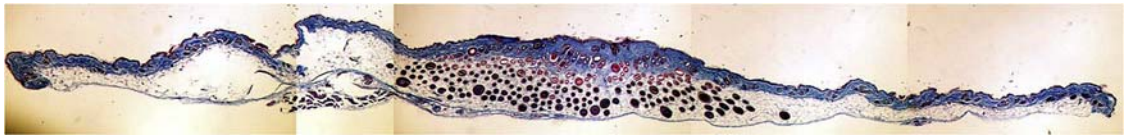


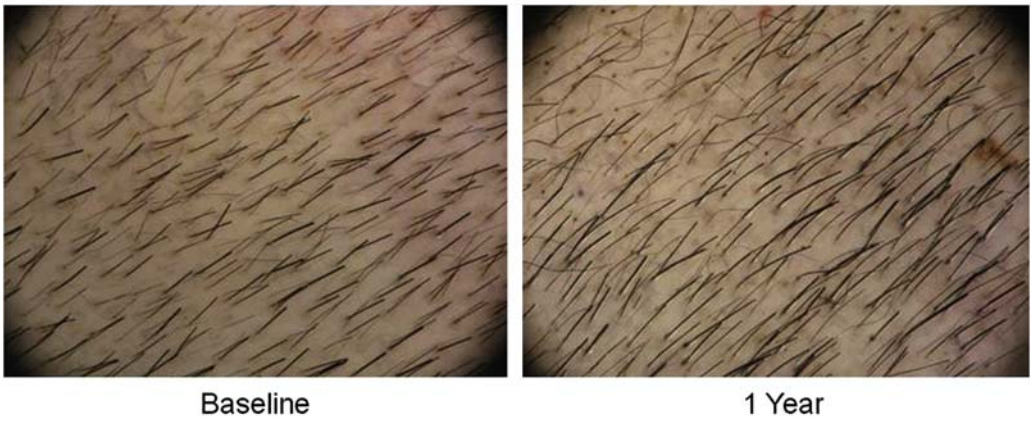
FIGURE 74.3 Injection of hypoxia-induced multipotent stem cell (HIMSC)-conditioned media subcutaneously into the C-57bl/6 mouse model induced an increased density of hair follicles at the site of injections.

NANOG, and KLF4 genes [31]. The expression of stem cell-associated proteins, including Nodal, Brachyury, Nestin, and Oct4, was also seen. It was also noted that genes were upregulated and surface markers were expressed, which are normally associated with follicular stem cells, including Lhx2, SOX 21, Nestin, NFATc1, and Krt 15 (Fig. 74.2).

Because of this finding, CM from the HIMSCs was utilized to study whether the composition could induce hair growth in the C57Bl/6 mouse model. Intradermal injections of the CM induced new hair growth in the treated mice and an increased number of hair follicles and hair follicle stem cells were noted in the treatment area (Fig. 74.3). No such hair induction was seen in the unconditioned media controls.

The CM was analyzed and shown to contain high levels of growth factors associated with the induction and increased endurance of anagen, including HGF, FGF-7 or KGF, VEGF-A, follistatin, angiogenin, and placental growth factor (PlGF-1 or PlGF). Given this composition, a proof-of-concept clinical study was performed to assess the ability of the HIMSC-CM, called hair-stimulating complex (HSC), to induce hair growth in men with male pattern baldness [32]. The clinical study was a double-blind, placebo-controlled, randomized, single site trial and was designed to evaluate safety of the HSC product and assess efficacy in stimulating hair growth. All 26 subjects tolerated the single, intradermal injection of HSC procedures well, and no signs of an adverse reaction were reported. Histopathological evaluation of the treatment site biopsies taken at 22 and 52 weeks posttreatment revealed no abnormal morphology, hamartomas, or other pathological responses. Treatment and control regions received temporary tattoos so that photographs at each follow-up time point could be taken in exactly the same region. Trichoscan image analysis of HSC-treated sites at 12 and 52 weeks showed significant improvements in hair growth over the placebo. At the initial 12-week evaluation period, HSC-treated sites demonstrated an increase in hair shaft thickness (6.3% $\pm$ 2.5% vs. $-$0.63% $\pm$ 2.1%; $P = .046$), thickness density (12.8% $\pm$ 4.5% vs. $-$0.2% $\pm$ 2.9%; $P = .028$), and terminal hair density (20.6 $\pm$ 4.9% vs. 4.4 $\pm$ 4.9%; $P = .029$). At the 1-year time point a statistically significant increase in total hair count ($P = .032$) continued to be seen. The fact that hair counts continued to increase over the 1-year period even after only one injection at day 0 supported the hypothesis that HSC stimulated a prolonged and sustainable anagen, a result that had not been reported in previous clinical hair growth studies. An example of such growth is seen in Fig. 74.4 where trichoscan data demonstrated a 123% increase in terminal hairs over the 52-week period.

To assess the efficacy and safety of an increased number of injections and a second dosing at week 6, a phase 1/2 study was conducted in Manila with Investigational New Drug (IND) approval by the Food and Drug Administration Philippines. The clinical study was a double-blind, randomized, two center trial in 56 subjects. All subjects tolerated the eight 0.1 cc intradermal injections at baseline and 6 weeks well, and no signs of an adverse reaction were reported. Blood and urine samples taken before and after each injection set showed no liver, kidney, or bone marrow toxicity. Trichoscan image analyses of treated and control sites were taken at baseline, 12, 24, 36, and 48 weeks. At the



Hair Count:	Baseline	12 week	1 year
HSC	214.5	224.5	324.5

FIGURE 74.4 One-time injection of hair stimulating complex (HSC) yielded significant new terminal hair growth in treated areas at 12 weeks and 1 year.

12-week time point, significant improvements in total ($P = .0013$), terminal ($P = .0135$), and vellus ($P = .033$) hair growth over baseline were seen, as was an increase in cumulative thickness density ($P = 026$). The primary efficacy endpoint of increased terminal hair at 12 weeks was met, with a 19.5% increase seen, a 49.5% increase over the same endpoint in our proof-of-concept trial. In addition, unlike currently approved products, HSC induced hair growth in the temporal recession, as well as vertex and mid-scalp regions, and was highly effective in men over 40 years of age, as well as in men in their 20s and 30s. At the 48-week time point there continued to be a significant increase in total hairs over baseline ($P = .028$). These results clearly demonstrate the safety and efficacy of intradermal injections of HSC in subjects with AGA and the benefit of a second treatment at 6 weeks to capture preanagen follicles that were too early in the cycle at week 0.

An additional clinical study was conducted on five men and five women in the United States under a physician IND. This "safe passage" study involved 20 (0.1 cc) injections at baseline, 20 at week 6, and 40 injections at week 12 and week 40. The injections were well tolerated and no serious adverse effects were reported. Some subjects reported an itching at the injection sites, which was resolved within 30 min. An increase in hair growth, as demonstrated by global photography, was seen in all patients, which included men with male pattern baldness, as well as women with hair loss associated with stress, chemical damage, and perimenopausal hormonal changes. Fig. 74.5A and B show results in two of the 10 patients treated.

To be able to do phase 2 dosing studies in men and to remove nongrowth factor-related impurities from the CM, HSC production was modified to include capture of the growth factors by a heparin sepharose column and the use of various filters to remove DNA. The six key growth factors in HSC that are associated with hair growth are measured by enzyme-linked immunosorbent assay before product release and sterility, bioburden, and a series of viral testing are performed. Cell potency assays are performed to assess the activity of follistatin, FGF-7/KGF, and the angiogenic factors in the product. The following is a summary of the proposed mechanism of action of HSC in the induction of hair growth.

A number of growth factors work synergistically to induce and maintain angiogenesis in the hair follicle. The hair follicle undergoes cyclic expansion and regression, which requires changing demands for vascular support, with perifollicular vascularization being seen during anagen and decreased vascularization associated with catagen and telogen. VEGF helps to mediate the induction of angiogenesis to provide the increased nutritional needs of hair follicles during rapid cell division in anagen [33]. In addition to increasing vascularization, VEGF induces the division of DPCs, which is a key step in the induction of hair follicle formation. Angiogenin is a soluble factor normally secreted by DP cells [34]. Angiogenin is a potent angiogenic factor that also promotes the stimulation of DP and ORS keratinocytes and the subsequent elongation of the hair follicle.

HGF is a multifunctional polypeptide that acts as a mitogen, motogen, and morphogen. In addition to promoting angiogenesis, it has been shown to stimulate follicle growth of human hair and follicle elongation by stimulating DP

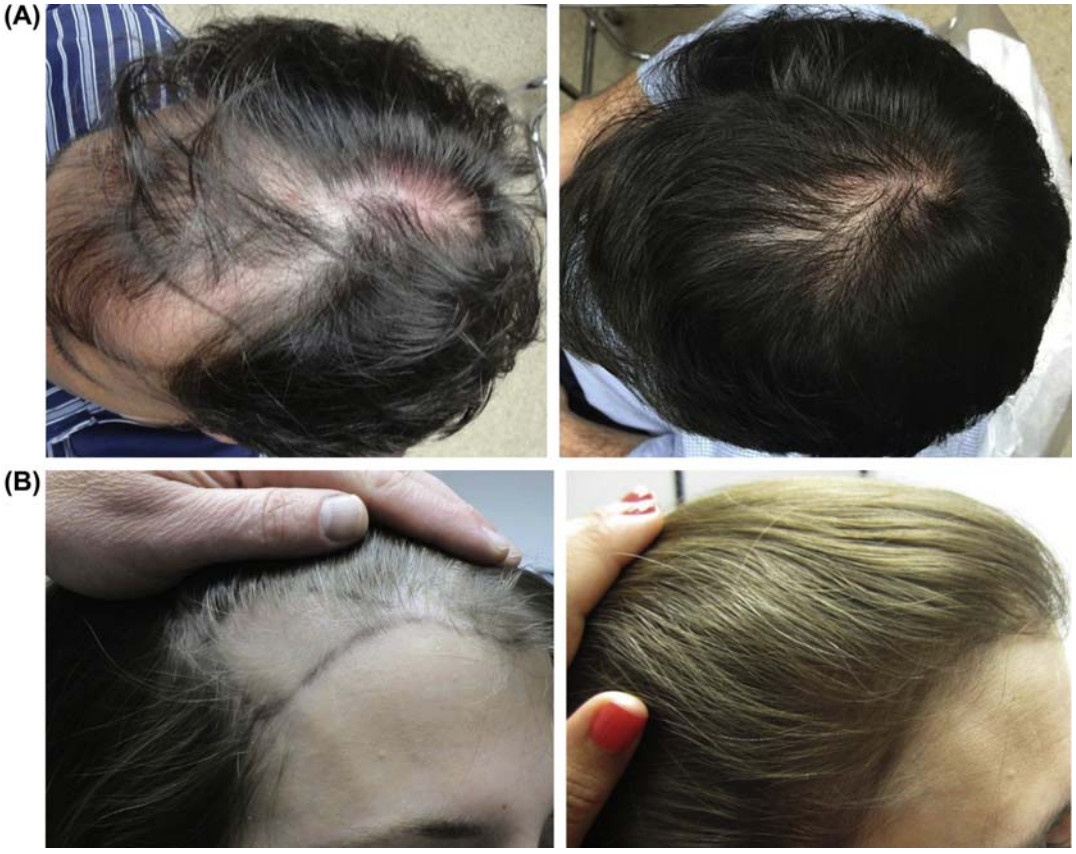


FIGURE 74.5 (A) Escalating doses of hair-stimulating complex (HSC) every 6 weeks induced cosmetically relevant new hair formation in a subject with male pattern baldness. (B) HSC induced new vellus hair formation within 18 weeks in the temporal recession region of a 38-year-old female with stress-induced alopecia.

cells and hair bulb-derived keratinocytes [35,36]. PlGF is a member of the VEGF subfamily and is important in promoting angiogenesis and vasculogenesis, particularly during embryonic development. PlGF has been shown to play a role in the promotion of hair growth by accelerating the growth of hair follicle cells and prolonging anagen hair growth [37]. The mechanism of action involves preventing hair follicle cell death by increasing levels of phosphorylated extracellular signal-related kinase and cyclin D1.

FGF-7/KGF is synthesized by skin fibroblasts and DP cells and has a mitogenic effect on skin keratinocytes. It is thought to be the most potent growth factor reported to date [38]. The KGF receptor is found on ORS cells and addition of KGF to these cells in organ cultures has significantly increased hair follicle cell proliferation [39]. In vivo the FGF-7/KGF secreted by DP cells causes both rapid proliferation of ORS cells and the resulting keratinocytes to migrate into the hair follicle shaft and form a new vellus hair or convert vellus hairs to terminal hairs [40].

Follistatin is found in all human tissues and organs and has the primary function of binding and bioneutralizing members of the TGF superfamily, with particular affinity for activin. It is an antagonist to both activin and BMP, both of which are involved in maintaining a slow cycle of stem cell proliferation in resting hair follicles [41,42]. Follistatin has been shown to be an important regulator of cell proliferation, differentiation, and apoptosis in hair follicle initiation and hair cycling. In AGA, androgens induce TGF-B1, which inhibits growth of the dermal papilla cells and keratinocytes [43]. Adding follistatin to dermal papilla cells helps to reverse this blockage, further strengthening the role of this activin antagonist in hair growth [44].

Naturally occurring growth factors work synergistically to promote proliferation, differentiation, and angiogenesis. The composition of growth factors in HSC works together to induce angiogenesis of the hair follicle for adequate nutrient support, as well as stimulating DP cells and ORS cells to proliferate to induce hair follicle elongation, cell migration into the hair shaft, and subsequent hair growth.

Given the early clinical results with HSC, a study was performed in Japan to see if it could be effective as a holding solution for follicular units implanted in hair transplant procedures [45]. Saline is the most common solution used to

preserve hair follicles for the period of time between removal and implantation. Surgeons have come to accept a large degree of hair loss, known as effluvium, from the transplants, and patients need to be patient for the months it takes to allow the transplanted hair to recover from shock and enter into the anagen cycle. As expected in the weeks following transplant, the investigator reported that transplanted hairs held in saline were lost to effluvium at the 6-week follow-up. Of the hairs held in the human cell conditioned media (hCCM) solution, however, most remained intact at this follow-up time point, reflecting not only the more hospitable environment created by the hCCM but potentially also the increased wound-healing capabilities of the material. Fifty-five percent of the follicles soaked in saline for up to 2 h during the transplant procedure showed hair loss, whereas only 10% of the follicles soaked in hCCM resulted in showing the effluvium response.

BIOENGINEERING A HUMAN HAIR FOLLICLE

A major challenge facing stem cell biologists is the bioengineering of an entire functional organ.

Since the hair follicle is a miniorgan it is an ideal candidate to utilize as a goal to be the first organ to be engineered. Formation of such a follicle would require simulating the series of molecular and cellular events that will recreate follicle morphogenesis. The steps will progress from the formation of dermal condensations, to epithelial invagination, to DP formation at the base of the follicle, to the arrangement of the various cell types and their morphogenesis, to the differentiation of hair shaft components, and then to actually demonstrate the creation of a hair. Creation of a functional hair follicle would not only be a major milestone in regenerative medicine, it would allow the creation of tissue-engineered skin for burn patients that would provide the critical functions of skin appendages in addition to providing a novel therapy for people with alopecia.

Early work studying how hair follicles are formed during early development resulted in the reconstitution of pelage hair follicles when a mixture of prepared cell suspensions from embryonic or neonatal mice was grafted onto the dorsal surface within a silicon chamber [46]. Zheng et al. [47] demonstrated that dissociated neonatal mouse epidermal and dermal cells could be reconstituted to form hair follicles. The de novo follicles formed in a sequence similar to that seen in embryonic development, showed follicular layering, formed a bulge region, as well as a sebaceous gland, and demonstrated appropriate biochemical differentiation markers and cycling. Isolated K15 enhanced green fluorescent protein-positive bulge cells showed stem cell properties and created new follicles. An additional important finding was that the ratio of epithelial and dermal cells injected affected the efficiency of the hair formation. The study showed that 5000 dermal cells and 2500 epidermal cells were necessary to produce a single follicle.

Ebama et al. [1] used a similar technique to attempt to create a functional human follicle. Cografting of neonatal foreskin human keratinocytes with murine DP-enriched cells produced structures resembling hair follicles with multiple epidermal cell layers and a well-keratinized inner region. The follicular structures expressed hair keratin markers, as well as markers associated with developmental stages, but lacked normal hair. These results were also repeated with adult keratinocytes. Neither the enriched DP population nor the epidermal fraction alone formed any follicles, further elucidating the importance of the epithelial−mesenchymal interactions in hair follicle regeneration.

Building on the research with the mouse and human-dissociated cells to form new follicles, Sriwirlyanont and colleagues worked to produce functional follicles in engineered skin [48]. To date the growth of hair appendages in tissue-engineered skin substitutes has not been accomplished in a reproducible manner predominantly due to the lack of trichogenicity in postnatal cells. In this study a chimera of human neonatal-cultured keratinocytes and murine-cultured DP cells was grafted orthotopically to full thickness wounds on athymic mice. Noncultured dissociated murine skin cells were used as the positive control and human cultured keratinocytes and fibroblasts without DP cells were the negative control. Neonatal murine-only skin substitutes produced external hairs with sebaceous glands, while chimeric skin substitutes formed hairs without the gland. The latter was associated with the upregulation of the LEF-1 hair-related gene and the downregulation of stearoyl-CoA desaturase, a sebaceous gland marker. No visible hairs or glands were formed with human-only skin substitutes. Results of this study illustrated that sebaceous gland formation is not a prerequisite for hair growth in tissue-engineered skin substitutes.

The regeneration of a human hair using epidermal and dermal components in ex vivo or mouse models has not been demonstrated to date.

Research by Rahmani et al. [49] has resulted in identifying a cell type that has furthered progress in human hair regeneration and may lead to generating new hair in the human scalp. To assess how DP cells are maintained in healthy follicles an in vivo fate mapping of adult hair follicle dermal sheath (DS) cells was performed. It was seen that a subset of DS cells is retained following each hair cycle and that these cells exhibit a self-renewal characteristic and are key in repopulating the DS and DP with new cells. Hair regrowth was retarded when the DS cells

were ablated and hair type specification was altered, supporting the hypothesis that the DS cells function to modulate normal DP function. This work identified a bipotent stem cell within the adult hair follicle mesenchyme and supported the approach of using the DS cells in the clinical setting.

A phase 1/2a study utilizing an autologous cell-based treatment for AGA has been completed by RepliCel Life Sciences and has demonstrated safety and effectiveness. Subjects with mild-to-moderate AGA as characterized by the Ludwig scale (female subjects) or Norwood Hamilton scale (male subjects) were enrolled in the study. After blood sample testing substantiated their general health, scalp biopsies were taken and sent to a cyclic guanosine monophosphate-compliant facility. Biopsies were dissociated and treated to isolate dermal sheath cup (DSC) cells, which were then expanded in culture. The 19 subjects returned to the clinic after the DSC cells were grown to numbers sufficient for transplantation. The process for creating and implanting the DSCs is shown in Fig. 74.6. Subjects were their own control and received medium alone (placebo) or replicated DSC cells in medium into areas of their scalp that were randomized by a predetermined blind. The primary protocol of the study was to assess local treatment sites for any abnormalities at the 6-month time point. The secondary endpoints at 6 and 24 months assessed both safety and efficacy. At the 6-month interim point the data collected showed no serious adverse reactions in any of the subjects. Thirty percent of the subjects experienced a burning sensation at the site of treatment, which was resolved within 24–48 h. At 6 months, several efficacy endpoints were successfully met as well. Significantly more patients demonstrated a greater than 5% increase in hair density at the DSC-treated areas, with density increases ranging from 5% to 19.6%. Increases were seen in total hair density, as well as vellus and terminal hair density. Additional clinical studies are currently under way in Japan and are being conducted by Shiseido Company, a world leader in aesthetic products. The clinical data to date support the importance of DS cells in hair growth, particularly with the cosmetically relevant increase in hair density.

Similar autologous cell-based studies have been performed with a combination of epithelial and dermal cells or dermal cells alone by both Intercytex Group plc and Aderans Research. Trials have consistently shown safety in all patients and signals of efficacy in a number of treated subjects.

In autologous cell-based trials a number of questions still remain to be answered. These include the mechanism of action and whether the injected cells actually develop a new follicle, become part of an existing follicle, or stimulate hair growth by releasing growth factors and exosomes that then increase growth in the targeted hair follicle. The

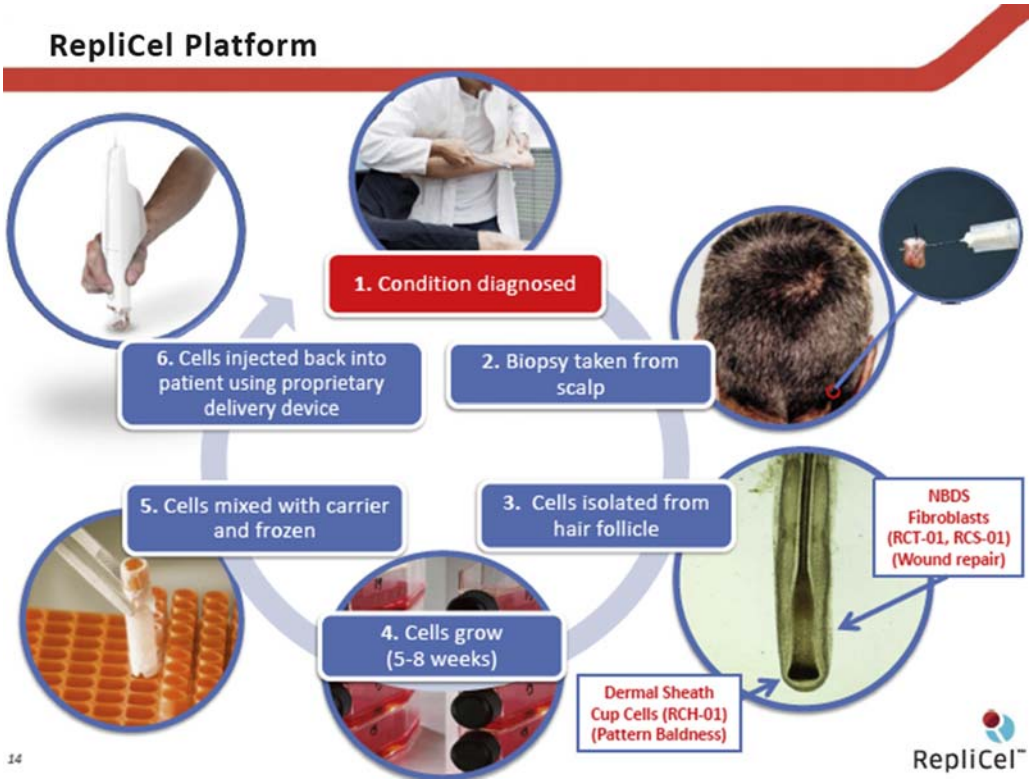


FIGURE 74.6 Collecting and processing dermal sheath fibroblasts for implantation for hair regrowth.

number of cells necessary to be utilized to stimulate hair growth is key, since the ratio of new hair follicles to donor follicles must be great enough to produce a commercially feasible product. Being able to measure and retain trichogenicity of the implanted cells is essential, as well as being able to understand whether a regenerating system in vivo has the ability to attract other cells from the body, as is seen with the recruitment of bone marrow cells in the process of normal wound healing, which should offer a real benefit in new organ formation.

SUMMARY

The ultimate goal in organ replacement regenerative medicine is the ability to create fully functional organs that have been damaged or destroyed by injury, aging, or disease. The hair follicle is an ideal miniorgan, which has been targeted to achieve this goal since it reforms itself throughout life through key interactions between epithelial cells and inductive cells from the mesenchyme. Whether success will be achieved by creating the follicle in vitro in complex three-dimensional culture systems or injecting a combination of cells and growth factors into the scalp for remodeling in vivo is still unknown. Studying follicular neogenesis in the embryonic environment has led to tremendous advances in understanding the complex signaling pathways and epithelial−mesenchymal interactions necessary to stimulate human hair growth. Research by several laboratories has underscored the powerful inductive ability of the follicular dermal cells. Although current cellular implant approaches have focused on autologous dissociated cells from the scalp, the potential of an allogeneic approach is very feasible. Dermal hair follicle tissue has successfully been transplanted between individuals without any sign of rejection [50]. In addition, it is believed that the hair follicle is an immune privileged location that does not express the major histocompatibility complex class 1 antigen that is associated with the rejection process [51]. To make progress in either autologous or allogeneic expanded cells for transplantation we must make progress with developing solutions for maintaining trichogenicity of the cells in culture. A very intriguing proposition is the utilization of iPS cells or other stem cells in bioengineering a human hair follicle. A number of stem cell types, including neural [52] and bone marrow-derived MSCs [53], have shown the capacity to form skin and hair when injected into a blastocyst. A product designed to engineer new hair follicles, consisting of inductive dermal cells and competent epithelial cells, is likely to be the first organ system to be successful in the clinic.

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Further Reading

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CHAPTER

75

US Stem Cell Research Policy

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INTRODUCTION

Since James A. Thomson and colleagues reported the isolation of pluripotent stem cells from human embryos in 1998 [1], stem cell research has received considerable public and policy attention. Because the isolation of embryonic stem cells involves destroying human embryos, some groups and individuals have opposed, or raised concerns about, some or all of the research on moral grounds. The opposition and concern have influenced a number of policies and laws at state, federal, and international levels. From 2001 until 2009, much embryonic stem cell research was ineligible for federal funding in the United States. In 2009, federal rules were relaxed somewhat, although federal funds still may not be used to create embryonic stem cells or to support research on cells taken from embryos created for research purposes, including by cloning. Despite these restrictions, embryonic stem cell research progressed into the 21st century in the United States using monies supplied by individual donors, charitable organizations, and states. After briefly discussing ethical and policy issues in adult and fetal stem cell research, this chapter will survey the current policy issues in embryonic and induced pluripotent stem cell (iPSc) research, beginning with federal and state funding policies, which will be compared to regulation strategies adopted in other nations active in the research, before considering oversight, commercialization, and ethical issues that arise as the research and technology move forward.

SOURCES OF STEM CELLS

Stem cells are special kinds of cells that can regenerate themselves and make new, more specialized cells. For the purposes of an ethics and policy discussion, stem cells can be divided into four kinds based on the source of the cells: adult stem cells, fetal stem cells, iPScs, and embryonic stem cells. In terms of ethics, politics, policy, and law, much depends on the source of the cells.

Adult Cells

"Adult" stem cells are derived from the somatic cells of adults and children (frequently taken from an umbilical cord). Although obtaining adult stem cells can raise some ethical issues, they are similar to those raised in other kinds of research involving human subjects, the most important of which is the requirement for free and informed consent [2]. Because the ability of competent adults to consent to research enjoys wide acceptance, somatic stem cell research has not been a major focus of ethical or political debate. It does, however, enter public consciousness as a less controversial, proposed alternative to embryonic stem cell research. Opponents of embryonic stem cell research cite clinical success of adult stem cells as evidence that research using embryonic cells is unnecessary [3,4]. However, many adult stem cell scientists insist on the importance of pursuing research on stem cells from both sources [5,6].

Fetal Cells

Pluripotent stem cells have also been extracted from the primordial reproductive tissue of aborted fetuses [7]. Any source of stem cells that relies on women undergoing elective terminations is likely to be controversial in the United

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States simply because of the ongoing debate over the morality and legality of abortion. Nevertheless, researchers have used tissue from aborted fetuses since as early as the 1930s and federal funds are currently available for this kind of stem cell research.

Although research involving human fetuses has been regulated in the United States since allegations of experiments on in and ex utero fetuses emerged in the early 1970s [8] (and around the same time as the Supreme Court's famous 1973 decision on the legality of abortion in *Roe v. Wade* [9]), it seems that these regulations do not apply to most stem cell research using fetal cells. Human subject regulations, promulgated in 1975, require extra protections where federally funded research involves pregnant women, fetuses, and human in vitro fertilization (IVF). Among other things, these regulations forbid researchers from having any involvement in a woman's decision to terminate her pregnancy or from offering gestational carriers financial inducements [10]. Although it has always been clear that these regulations applied to in utero fetal research, there has been some uncertainty as to whether the regulations apply also to research that, like stem cell research, uses cadaveric fetal tissue [11]. In March 2002, the Office for Human Research Protections issued a guidance for research involving fetal stem cells in which it states that the research will only be subject to federal human subject protections where it involves "a living individual," which would exclude fetal stem cell research unless the formerly pregnant woman is involved in the research [11].

Likewise, the 1993 federal legislation on fetal tissue transplantation research likely does not apply to stem cell research using fetal cells unless the research also involves transplanting the cells into humans. Similar to the restrictions imposed by the federal human subject regulations, this legislation stipulates that no alteration in the timing, method, or procedures used to terminate the pregnancy should be made solely for the purposes of obtaining the tissue, nor can tissue be paid for or taken without the consent of the gestational carrier [12]. This legislation reappeared in public discourse in 2015, when the reproductive health care provider Planned Parenthood was accused of illegally profiting from fetal tissue donated to research [13]. Under the 1993 law, centers such as the accused California clinic can be reimbursed for the cost of obtaining, storing, and processing fetal tissue, but cannot be paid for the donation itself. Although 12 state inquiries found no evidence of illegal activity on behalf of Planned Parenthood, the organization stopped obtaining reimbursements for fetal tissue in October 2015 [14].

Even though both the federal regulations and the fetal tissue transplantation legislation likely do not apply to most fetal stem cell research, practices similar to those required by these laws (and lessons learned from cases that arise from their interpretation) will likely be considered by Institutional Review Boards (IRBs) reviewing fetal stem cell research.

Various states also have laws affecting fetal stem cell research, which will apply to all researchers in those states regardless of their funding source. For example, five states ban research involving aborted fetuses and 12 states ban paying for fetal remains [15]. Despite fetal stem cell research's intimate connections with fetal tissue research and the controversial practice of induced abortion, this kind of stem cell research has seldom been the subject of public debate.

Embryos

Instead, debate has consistently focused on research that uses stem cells extracted from 4- to 7-day-old human embryos, referred to broadly as human embryonic stem cells (hESCs). Because any single cell in the very early human embryo can develop into a whole fetus, it is thought that embryonic stem cells have the potential to develop into almost any cell type and repair damaged or diseased parts of the human body. The therapeutic potential of embryonic stem cells therefore is thought to be enormous, but so is the moral peril because extracting these stem cells generally necessitates destroying the embryo. For this reason, the research has been vigorously opposed by many individuals and groups, including (but not limited to) those who consider the early embryo to be a person or, if not a full person, an entity of such sufficient moral significance that it should not be created for, or destroyed in, research.

Embryonic stem cells can be derived from embryos created by two mechanisms: IVF or cloning. IVF embryos are created through the combination of an egg and sperm cell in a lab. Embryos produced through IVF may have been produced for reproductive uses but then determined no longer to be clinically necessary (so-called "spare" or "surplus" embryos), or they may have been produced specifically for research use. This difference in the reasons for creating the embryos is worth understanding. Creating embryos for research use is often opposed on the grounds that it is wrong to create human life for the purposes of destroying it [16]. Research use of spare embryos, on the other hand, has received more support on the ground that the embryos would be destroyed anyway.

Whether the intention of the original creator of the embryos is sufficient reason for permitting research on spare embryos but not on embryos created for research use has been questioned [17,18]. Even if this moral issue can be

resolved, spare embryos may not be satisfactory as the sole source of embryos for stem cell research because there may not be sufficient numbers available to meet demand. A 2003 survey of US fertility clinics reports that of the more than 400,000 embryos in frozen storage in the United States, only 2.8% have been donated to research [19]. Other researchers have argued that these frozen embryos will not be genetically diverse enough for therapeutic purposes [20]. Whether for these reasons or others, the US National Academies, which issued guidelines for embryonic stem cell research in 2004 (discussed in more detail later), proceeded on the basis that researchers could use spare embryos and embryos created for research use, including by cloning.

The arguments against creating life purely for research use are also used to argue against using cloning techniques to generate human embryos. Currently, cloned embryos are created through a technology called somatic cell nuclear transfer (SCNT) (SCNT was used in the cloning of Dolly the Sheep in 1997 [21]). In ethics and policy discussions, cloning is usually broken down into two types based on the end goal of the cloning: therapeutic or research cloning and reproductive cloning. Therapeutic cloning uses SCNT to create cloned cells that will only be used in vitro, including embryos or somatic cells from which hESCs or iPScs can be derived, respectively. In therapeutic cloning, cloned embryos are not implanted for reproduction, and are discarded after stem cells have been derived [22]. Reproductive cloning is the creation of a cloned embryo through SCNT with the goal of creating a new living person. While therapeutic cloning destroys embryos, reproductive cloning would, if successful, result in the birth of a cloned human. Therapeutic and reproductive cloning are treated differently in policy in the United States and internationally.

Induced Pluripotent Stem Cells

In 2002, Catherine Verfaillie and colleagues announced that they had isolated multipotent adult progenitor cells from bone marrow [23]. Then, in 2006, scientists in Japan and the United States announced the creation of pluripotent stem cells from adult skin cells [24,25]. These iPScs are human somatic cells reprogrammed to develop into nearly every human cell type, and are believed to be functionally very similar or identical to embryonic stem cells. Many consider these somatic cell-based lines to be an ethically simpler alternative to embryonic stem cells that could satisfy the same scientific and clinical needs. In the past decade, iPSc technologies have contributed considerably to research on human diseases, drug screening, and, with the introduction of gene editing, offer a virtually unlimited supply of human cell lines to research efforts [26].

Debates continue regarding the scientific differences between hESc and iPSc lines, and the significance (if any) of the ways in which they vary [27,28]. Due to the self-renewing quality of both cell types, hESc and iPSc may introduce a risk of neoplasm and tumor formation if the cells are not engineered properly. As such, hEScs were only approved in clinical trials in 2009, and iPScs have yet to be used clinically [29,30]. While embryonic stem cells frequently garner ethical attention surrounding the destruction of embryos, pluripotent stem cells have raised separate ethical considerations, mainly stemming from their creation by human cloning technologies.

At the time of writing, research remains active in both the embryonic stem cell and iPSc domains. While future developments could shift research toward exclusive use of iPScs, past and current state, federal, and international policies have centered primarily on embryonic stem cell research.

UNITED STATES FEDERAL AND STATE STEM CELL POLICY

History of US Stem Cell Law and Policy

Federal policies affecting human stem cell research include those targeted specifically at the research, or aspects of it, and more general policy that has implications for stem cell research. In both cases, most of these policies affect hESc research.

An example of policy that predates the derivation of hEScs is federal regulation of research involving human embryos, which began in the 1970s. Following the 1973 decision of the Supreme Court in *Roe v. Wade*, the United States Department of Health, Education and Welfare (DHEW) (a predecessor to the Department of Health and Human Services [HHS]) placed a moratorium on research with living human embryos. In 1974 Congress followed suit, creating their own moratorium on federal funding for research on embryos and embryonic tissue. While the DHEW moratorium was lifted in 1975, a de facto Congressional ban remained, and was translated into legislation in 1995 with the passage of the Dickey–Wicker Amendment [31,32]. The amendment specifies that no HHS (which includes the National Institutes of Health [NIH]) funds can be used for the creation or destruction of a human embryo for research

purposes. Since 1996, this policy has appeared as a rider to the annual appropriation acts for the Departments of Labor, Education, and Health and Human Services. Since then, no research on human embryos themselves, including the derivation of stem cells and the creation of human embryos through cloning, has been supported with federal funds. Instead, funds from states and private sources have been used to create embryonic stem cell lines. Once those lines have been created, federal funding has been available for research on them (see later). While the terms of this funding have varied by administration, Presidents Clinton, Bush, and Obama formulated policies to allow for federal funding of research on hEScs, within the bounds of the Dickey–Wicker Amendment. Unless Congress removes this provision from HHS appropriations, or until embryonic stem cells (or their equivalent) can be extracted without destroying or harming human embryos [33], federal funding cannot be made available for the critical derivation step in the research.

Following the announcement of derivation of hEScs in 1998, President Clinton developed a policy to permit use of federal funds to study embryonic stem cells without contravening the Dickey–Wicker Amendment [2]. Under the policy, federal funds could be used for research on embryonic stem cells *after* they had been extracted from the embryo, but the funds could not be used for the crucial extraction step in the process. In opposing President Clinton's policy, some members of Congress complained that although it did not pay for embryo destruction, it nevertheless encouraged research that required the destruction of human embryos [2]. The basic reasoning in President Clinton's policy—that extraction of stem cells from embryos is a separate step from research on those cells once extracted—has persisted in the policies of Presidents Bush and Obama since, and was tested in a federal court in 2010. However, President Clinton's policy itself was not able to be implemented before his presidency ended, and the federal policy was altered dramatically in 2001, when President George W. Bush announced that he would limit federal funding to the study of embryonic stem cells that were already in existence at the time that his policy was announced (9 p.m. Eastern Standard Time on August 9, 2001). Research on newly derived cell lines would not be permitted with federal money. Other conditions included that the cells must have been extracted from "spare" embryos that were donated with the informed consent of the donors, to whom no financial inducements may have been offered [34]. To facilitate research on existing embryonic stem cells that met these criteria, the NIH set up a stem cell registry shortly after the policy was announced listing embryonic stem cell lines eligible for use in federally funded research and available for shipping. In his 2001 address, President Bush stated that more than 60 embryonic stem cell lines met his criteria, but for a variety of reasons only 22 lines were consistently listed in the registry as available for shipping.

In explaining his policy, President Bush explicitly referred both to scientists' beliefs in the enormous therapeutic potential of embryonic stem cell research and to his own belief in the value of embryonic life. Of embryonic stem cell research, he noted: "At its core, this issue forces us to confront fundamental questions about the beginnings of life and the ends of science. It lies at a difficult moral intersection, juxtaposing the need to protect life in all its phases with the prospect of saving and improving life in all its stages." He called himself "a strong supporter of science and technology" but also noted that he believes "that human life is a sacred gift from our Creator" [35].

If President Bush intended his policy as a compromise between the value of scientific research and the value of embryonic life, it was one that left many scientists, disease groups, and others unsatisfied. Substantial criticism was directed at the 2001 policy. Those who oppose any research in which human embryos are destroyed argued that the federal funding restrictions needed to be supplemented with a nationwide ban on creating embryos for use in research, including by cloning [2,36]. A more vocal opposition called the 2001 policy overly restrictive. Their arguments included that the President overvalued embryonic life (that it is not more important than research aimed at treating disease) [2], that the policy was arbitrary and inconsistent [2], that the cell lines in the NIH registry were of poor quality and inappropriate for long-term use [37], and that the policy harmed American science by encouraging scientists to focus on research for which federal funding is available or to move to other countries to conduct their research [2].

During the Bush presidency, stem cell research was an important and in many cases partisan political issue. Federal policy was debated during the 2004 presidential campaign [38] and was a major issue at the Democratic National Convention, where Ronald Regan Jr., son of former Republican President Ronald Regan, gave a speech calling for greater government support of embryonic stem cell research. State policies to fund hESc research ineligible for federal support (described later in the chapter) were in some cases motivated by disagreement with President Bush's 2001 policy.

Other political activity, however, was bipartisan, including two 2004 letters—one signed by over 200 congressional representatives and the other signed by nearly 60 senators—asking President Bush to relax federal funding restrictions on embryonic stem cell research. Similar bipartisan support was expressed for the Stem Cell Research Enhancement Act, introduced in 2005 and 2007, which would have provided federal support for hESc research using embryos created for IVF, no longer clinically necessary and donated with informed consent [39–41]. Both attempts

were passed by the House and Senate, but vetoed by President Bush (an override was unsuccessful in 2005 and not attempted in 2007). Republican politicians who supported changes in federal policy included Senator Orrin Hatch, who mixed opposition to abortion with support for embryonic stem cell research provided it used only spare embryos [42]. Although the Stem Cell Research Enhancement Act was never passed, its language and motivations contributed to a shift in federal policy at the commencement of the Obama administration.

Current US Stem Cell Research Policy

Federal Policy

At the time of writing, US policy for embryonic stem cell research is contained in President Obama's March 9, 2009 Executive Order [43], the NIH Guidelines on Human Stem Cell Research [44], and any applicable state laws [45]. The 2009 Executive Order and NIH guidelines establish the criteria for embryonic stem cell research that uses federal funds. They do not apply to research conducted using nonfederal monies, such as might be supplied by an individual donor, a company, a charitable organization, or a state government. Nevertheless, because much basic biomedical research is traditionally funded by the federal government, the federal funding policies have a significant impact on embryonic stem cell research in the United States.

Executive Order 13505 does little more than revoke the policy announced in 2001 by President George W. Bush, state that the NIH may fund research using embryonic stem cells, and request that they issue new guidance for such research. The particulars of the new federal funding policy are to be found in the NIH Guidelines on Human Stem Cell Research, released in 2009. Under these guidelines, federal funding may be used to study embryonic stem cells derived from embryos "that were created using in vitro fertilization for reproductive purposes and were no longer needed for this purpose" (sometimes referred to as spare, surplus, or leftover embryos) and that were donated by the individuals who sought reproductive treatment. The donors must have given their voluntary written consent for the embryos to be used in research, and documentation must be available to show, among other things, that the donors received no cash or in-kind payment in exchange for making the donation and that there was a clear separation between the decision to create the embryos for reproductive purposes and the decision to donate. The guidelines also establish a registry of cell lines that meet federal funding criteria and describe procedures for establishing eligibility for funding of lines that are not already on the NIH registry. Because of the continued applicability of the Dickey–Wicker Amendment, federal funding is not available for the derivation of stem cells from embryos or research on cells taken from embryos that were created for research purposes, including by cloning [44].

Although considerably less controversial, President Obama's Executive Order and the NIH guidelines that followed have also been criticized for being too permissive by those opposed to embryonic stem cell research and too restrictive by those who believe federal funding should be available for research on stem cell-extracted embryos created for research purposes, including by cloning [46].

Overall, stem cell policy under President Obama has received little political attention. The most notable opposition came by way of the courts, just months after the announcement of Obama's executive action. A suit was brought against the Obama administration (specifically, HHS Secretary Kathleen Sebelius) by two adult stem cell researchers, James Sherley and Theresa Deisher, who argued that federal policy violated the spirit of the Dickey–Wicker Amendment by funding research that relies on the destruction of human embryos. The case succeeded initially in the federal district court for the District of Columbia [47]. But after a series of appeals, the US Court of Appeals for the District of Columbia Circuit overturned the decision in 2012, citing no wrongdoing or violation of Dickey–Wicker in the NIH's interpretation of the guidelines [42]. In 2013, the Supreme Court refused to hear the case, decidedly upholding NIH's 2009 guidelines and the limited federal funding of hESc research [48] (Figs. 75.1 and 75.2).

State Policy and Private Funding

In response to President Bush's 2001 hESc research funding policy, advocates lobbied both state governments and private parties to provide funds for the research. The funding helped propel the research forward. Harvard University's Douglas Melton and colleagues announced in 2004 that they had derived 17 new embryonic stem cell lines with support provided by the Juvenile Diabetes Research Foundation, the Howard Hughes Medical Institute, and Harvard University [49]. According to a 2005 special report in *Scientific American*, about $200 million of private money was spent on US stem cell research annually during the Bush administration [50]. We could find no reports of whether or how private funding of hESc research has changed since 2009. However, private foundations continue to contribute heavily to hESc research despite the increased federal funding under the Obama administration [51].

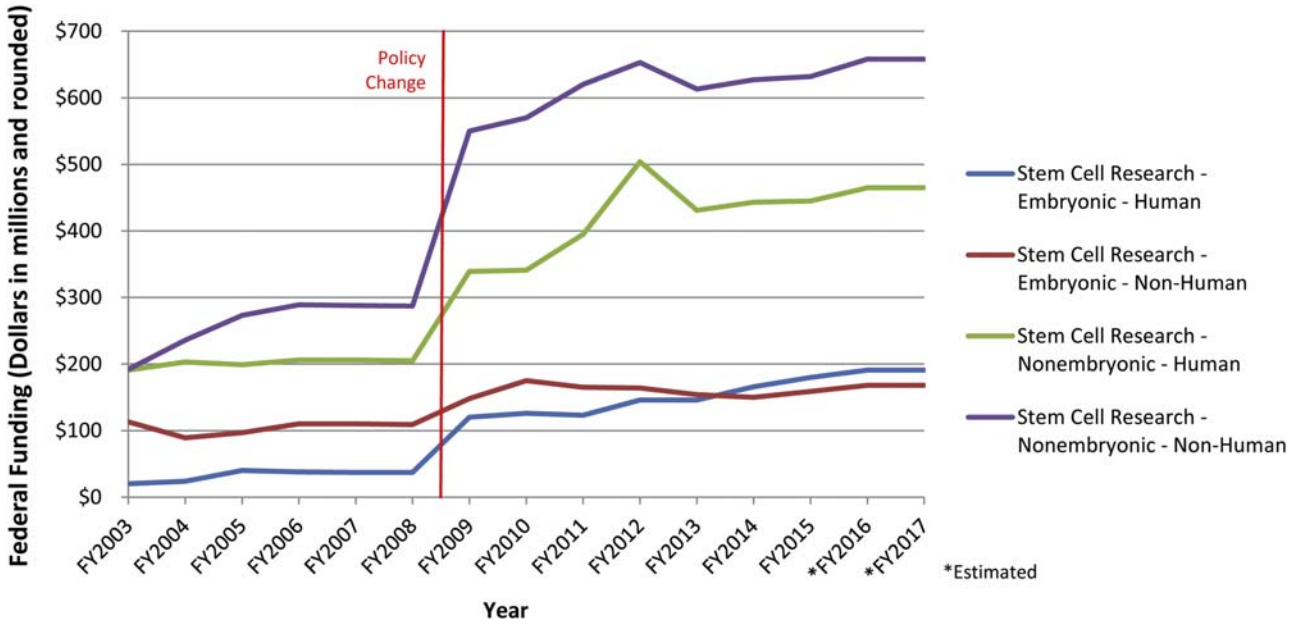

FIGURE 75.1 **Federal funding for embryonic versus nonembryonic stem cell research (FY2003–FY2017).** Changes in federal funding for embryonic and nonembryonic human and nonhuman stem cell research between the years 2003 and 2017. The *red line* denotes the shift from the Bush administration to the Obama administration, at which time US federal stem cell research and funding policies changed significantly.

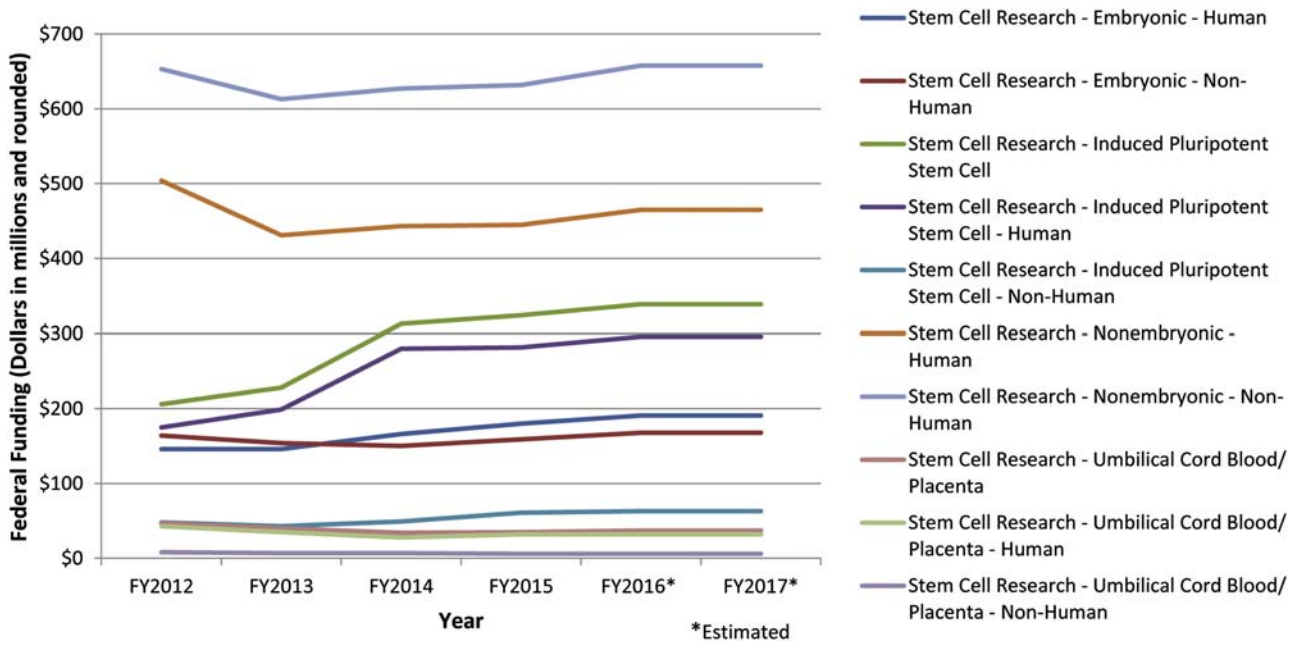

FIGURE 75.2 **Federal funding for stem cell research by type (FY2012–FY2017).** Federal funding allocations for stem cell research by type throughout the Obama administration (postfunding policy shift, 2012 through 2017). *Data courtesy of National Institutes of Health Research Portfolio Online Reporting Tools. Estimates of funding for various research, condition, and disease categories (RCDC); February 10, 2016. https://report.nih.gov/categorical_spending.aspx.*

In response to President Bush's hESc research policy, states also moved to fund stem cell research, as outlined in Table 75.1. In 2004, New Jersey became the first state to announce funding for local stem cell researchers, creating the Stem Cell Institute of New Jersey and the New Jersey Stem Cell Research Grant Program [52]. This program, as well as one in Illinois, has since ended, but funding continues to be available in other states, including New York, Connecticut, Maryland, Massachusetts, and Wisconsin [53].

TABLE 75.1 Human Embryonic Stem Cell Research Laws and Funding, by State

State	hESc Research Legal	Funding for hESc or cloning research	Derivation from Excess IVF Embryos	Cloning (SCNT) Legal	
				Therapeutic / Research	Reproductive
Alabama					
Alaska					
Arizona		Cloning: R and T			
Arkansas					
California					
Colorado					
Connecticut					
Delaware					
Florida		[a]			
Georgia					
Hawaii		Cloning: R			
Idaho					
Illinois		*Program Ended*			
Indiana	*	Cloning: R and T			
Iowa					
Kansas	*				
Kentucky					
Louisiana		Cloning: R and T			
Maine					
Maryland					
Massachusetts					
Michigan		Cloning: R and T			
Minnesota	[b]				
Mississippi					
Missouri		Cloning: R			
Montana					
Nebraska		hESc Derivation, Cloning[c]			
Nevada	[d]				
New Hampshire					
New Jersey		*Program Ended*			
New Mexico					
New York					
North Carolina					
North Dakota					
Ohio					
Oklahoma		[e]			
Oregon					
Pennsylvania	[f]				
Rhode Island					
South Carolina					
South Dakota					
Tennessee					
Texas					
Utah					
Vermont					
Virginia					
Washington					
West Virginia					
Wisconsin					
Wyoming					

hESc, Human embryonic stem cell; *IVF,* in vitro fertilization; *SCNT,* somatic cell nuclear transfer.

Key:

Green: Permitted by Law

Red: Prohibited by Law

Grey: Not considered in law - implicitly permissible, not governed by regulations or guidance

***** *Adult and fetal stem cell research are permitted, embryonic is not*

Continued

TABLE 75.1 Human Embryonic Stem Cell Research Laws and Funding by State—cont'd

State Specific Notes:

[a] *Amendments both allocating and prohibiting state funding for stem cell research have been introduced in Florida, neither have passed into law.*

[b] *Minnesota law prohibits use of living human conceptus (any human organism from fertilization through the first 265 days thereafter) for any type of scientific, laboratory research or other experimentation. It is unclear whether embryonic stem cells fall under this definition: while the publically funded University of Minnesota Stem Cell Institute is permitted to use embryonic stem cell lines (from embryos created for IVF and not necessary for reproduction), no grants for hESc research have been issued in the state.*

[c] *Nebraska limits embryonic stem cell research with state funds; restrictions apply to state healthcare funds provided by tobacco settlement dollars. No state facilities or funds can be used to destroy or create an embryo for the purpose of research (no derivation of stem cells or SCNT). Thus, derivation of hESc and use of SCNT can be conducted with public funds only with cell lines developed elsewhere. Both hESc derivation and SCNT are permitted using private funds.*

[d] *A 2005 veto indirectly allows Nevada state funds to be used for research on human embryonic stem cell lines that existed before August 2001 (in accordance with the Bush Policy).*

[e] *In 2015, the Oklahoma House passed a bill criminalizing embryonic stem cell research. The bill did not progress through the Senate and was not passed into law. State funding is allocated only to non-embryonic stem cell research, but funding is not explicitly forbidden by law.*

[f] *Like Minnesota, Pennsylvania law prohibits "knowingly performing nontherapeutic experimentation upon any unborn child […from fertilization until birth]." However, it does not explicitly ban embryonic stem cell research, which has been conducted at the University of Pennsylvania.*

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Thus far, however, the largest state initiative has been in California [54]. In November 2004, voters in California supported a proposition to allocate $3 billion over 10 years to embryonic stem cell research. The initiative, known as Proposition 71, authorized the state of California to sell $3 billion in general obligation bonds to provide funding for stem cell research and research facilities in California. Under the proposition, the funds have been distributed as grants and loans to California-based institutions by the Californian Institute for Regenerative Medicine (CIRM, established 2006), which also established regulatory standards for the research [55]. Critics of the initiative called it fiscally irresponsible given the state's economy and other health and research needs, and argued that the institute as structured lacked accountability [55]. Nevertheless, 59% of voters approved the measure. In 2015, California launched CIRM 2.0, described as a "radical overhaul" of the agency's operations, systems, and programs with an emphasis on speed, partnerships, and patient preference. In 2015, the state provided $135 million to 47 grants and 252 programs supporting stem cell research [56].

STEM CELL RESEARCH GUIDELINES

The National Academies of Science

The very limited federally funded hESc research program combined with increased interest in hESc research by states and private funders created a situation in which research was being funded by a variety of funders around the United States without the benefit of national rules or guidelines. In 2004, issues surrounding creation, use, and donation of hEScs were taken up by a panel convened by the US National Academies of Science (NAS), a private, nonprofit organization whose mission is to advise the nation on issues in science, engineering, and medicine. While they do not carry the force of the law, in general the National Academies' reports and recommendations are very influential and its *Guidelines for Human Embryonic Stem Cell Research* [57] received significant media attention when they were first released in April 2005. The 2005 guidelines (and their 2007 and 2008 revisions) were instrumental templates for state legislatures and institutions as they developed their own funding and research policies.

They were adopted, at least in part, by most major US research institutions [58]. While the guidelines are not the first document to speak to the conduct of embryonic stem cell research in the United States, at the time they were formulated it was not clear whether any of the previous guidance applied to contemporary hESc research because previous guidance was formulated under President Clinton but never implemented and effectively revoked by President Bush, or was simply too restrictive to meaningfully guide institutions and researchers that had made the decision to move forward with the research [59]. The guidelines were therefore received as filling a policy vacuum.

President Obama's 2009 Executive Order and the subsequent guidelines released by the NIH overrode the NAS guidelines to a certain extent. In 2010, the National Academies released a significantly amended set of guidelines, intended to be interpreted in tandem with the NIH's regulations [58]. The committee noted that non-NIH guidelines (such as its own) would continue to be important in the areas not eligible for federal funding, and thus not governed by the NIH. Some of these areas include the derivation of stem cell lines (ineligible for federal funding under Dickey−Wicker), research on hESc lines that were derived from embryos produced from some source other than IVF for reproductive purposes (including IVF for research purposes and SCNT cloning methods), and the wider range of experiments on chimeras. In the areas in which the NIH guidelines do overlap, NAS recommended that the NIH guidelines supersede its own. Where research falls outside of the scope of the federal guidelines, NAS generally recommends that the research is "permissible only after additional review and approval." This research includes generation of hEScs by whatever means and research involving the introduction of hEScs into animals other than humans and primates at any stage of development. NAS continued to recommend that research involving in vitro culture of any intact human embryo for longer than 14 days, and the introduction of hEScs into nonhuman primate blastocysts or non-hEScs into human blastocysts should not be permitted at this time.

The committee that drafted the original NAS guidelines was asked to consider the use and derivation of stem cells from embryos originally created during fertility treatment, embryos created using donated eggs and sperm, and cloned embryos. It therefore did not engage in the debate over whether it is morally permissible to destroy human embryos in research, as is required for derivation of embryonic stem cells. It also did not consider whether there is a moral difference between research that uses spare embryos and research that uses embryos created specifically for research purposes, including by cloning. The acceptability of these sources of stem cells was assumed.

Overall, the NAS committee recommended banning very little scientific activity. Instead, it recommended institutional review of protocols, oversight of the involvement of egg, sperm, and embryo donors, establishment of stem cell banks, and documentation of research activity. Two recommendations were particularly significant. First, the committee recommended that much embryonic stem cell research be subject to a mixture of local and national oversight. Local oversight would occur at each institution engaged in embryonic stem cell research, which would establish an Embryonic Stem Cell Research Oversight (ESCRO) committee to oversee all issues related to the derivation and use of embryonic stem cells, review all proposals for scientific merit, maintain records of research that takes place at the institution, including registries of new cell lines, and educate investigators. As noted by the 2010 amendment, many stem cell institutions created ESCRO committees. While the 2009 NIH guidelines did not require such a board, the National Academies maintains the importance of ESCRO committees in consulting and training roles at all institutions conducting stem cell research. ESCRO committees remain especially important at centers conducting nonfederally funded hESc research, which is not subject to the same federal oversight or regulation. In many cases, local IRBs provide additional oversight. Even though much embryonic stem cell research does not strictly speaking need to go before an IRB, the NAS committee recommended that the procurement of egg, sperm, and embryos should always be reviewed by an IRB, regardless of the applicability of federal regulations, and that IRBs never waive the requirement for informed consent from a person donating cells, eggs, sperm, or embryos to research, even where the federal human subject research regulations provide for such a waiver.

The other significant set of recommendations in the 2005 NAS guidelines addressed the involvement of egg, sperm, and embryo donors. In line with much guidance, law, and regulation around the world, the NAS guidelines recommended requiring consent from embryo donors for research use of those embryos. They went beyond previous US guidance, however, by extending this requirement to egg and sperm donors, including when the embryo was originally created for fertility purposes. At the time of the recommendation, gamete donors were asked to consent to reproductive use of their gametes, but were not generally asked to consent also to subsequent research use of any embryos their gametes were used to produce [60]. This NAS committee noted that this requirement might rule out the use of some embryos already created for fertility purposes that are now in frozen storage. The most recent iteration of the guidelines specifies that "written agreement at the time of gamete donation that one potential use of the blastocysts and/or morulae is embryo research will constitute sufficient consent," meaning that consent specifically to hESc research is not needed [58].

On the issue of compensating egg, sperm, and embryo donors, the NAS guidelines noted the arguments in favor of compensation: paying egg and sperm donors is routine in the US fertility context, and many Americans

participating in other kinds of research are offered financial inducements to secure their participation. They acknowledged that arguments for compensating egg donors are particularly strong: "the invasiveness and risks of the procedure suggest that financial remuneration is most deserved, but at the same time there is a greater likelihood of enticing potential donors to do something that poses some risk to themselves" [58]. These arguments notwithstanding, the National Academies committee followed previous US guidelines and guidelines and laws from many other nations in recommending that egg donors be reimbursed only for "direct expenses," and that no payment whatsoever be offered to sperm or embryo donors. They did allow, however, for reimbursement of fertility clinics for costs, including professional services, associated with obtaining consent and collecting eggs, sperm, or embryos.

In addition to cash payment, the NAS guidelines recommended against compensation in kind. Donors are not to receive any benefit from their donation, including "personal medical benefit" (excepting autologous transplantation, where the donor receives stem cells derived from his or her donation). This rule would prevent a kind of egg- or embryo-sharing arrangement whereby women or couples receive cheaper or free fertility treatment in exchange for donating a portion of their eggs or embryos to stem cell research. Similar arrangements exist in the fertility context, where women or couples receive a discount if they donate some of their eggs to others undergoing fertility treatment. This arrangement would help make fertility treatment available at a lower cost, but it would also more quickly exhaust the woman's or couple's supply of eggs or embryos, thereby possibly reducing their chances of achieving pregnancy [61]. In 2008, NAS's Human Embryonic Stem Cell Research Advisory Committee revisited the issue of donor compensation. The committee retained its prohibition on compensating embryo donors and discussed the arguments for and against compensating gamete donors. The guidelines were amended to make explicit that "actual lost wages" qualify as direct expenses for which gamete donors may be compensated, but additional payments are still not permitted [62].

International Society for Stem Cell Research

In 2006, the International Society for Stem Cell Research (ISSCR) released its first set of guidelines for embryonic stem cell research [63]. ISSCR is the world's largest professional organization for stem cell scientists, and is not tied to specific countries or national policy agendas. The guidelines were revised and expanded in 2016 [64].

As can be seen in Tables 75.2 and 75.3, in many ways the updated ISSCR guidelines are very similar to those of the National Academies. Like NAS's recommendation to form institutional oversight committees, ISSCR recommends formation of Embryo Research Oversight committees. However, under the ISSCR guidelines, these committees could operate at not only an institutional level, but could also provide local, regional, national, and international oversight. The ISSCR guidelines recommend similar standards for informed consent and the reimbursement and payment for embryos and other biological materials, and, notably, agree with the NAS that informed consent must be collected from all gamete donors. The scope of technologies and research uses covered by the ISSCR guidelines is, like in the NAS guidelines, quite broad. Both sets of guidelines address (and by extension apply to) many more kinds of research than are covered by the NIH guidelines, including derivation of cells from research embryos. Nonetheless, ISSCR takes at times a more conservative approach to novel technologies, research types, and sources of embryonic stem cell lines than the NAS guidelines. For instance, their guidelines propose a more robust review for the derivation of new stem cell lines and for the use of cells derived from research embryos [64].

Likely because they are at the time of writing the most recently published set of guidelines, the ISSCR's recommendations address the broadest scope of technologies, including gene editing of nuclear genomes of human sperm, eggs, or embryos, mitochondrial replacement therapy, and the creation and use of human totipotent cells [64]. The potential of these technologies has expanded considerably in the years since the publication of the NIH guidelines and NAS's final 2010 report. While not specific to the American policy context, the ISSCR's recommendations provide critical insights into many developing uses of embryos and embryonic stem cell lines.

INTERNATIONAL COMPARISONS

Biomedical research is an international undertaking and stem cell research is no exception. The governments of nations active in embryonic stem cell research employ a variety of regulation strategies, most of which include some restrictions on and oversight of the research while still allowing it to move forward. No single regulatory approach has prevailed internationally, although some patterns have emerged. For instance, many nations use national legislation to regulate stem cell research, often requiring oversight by a national stem cell research committee

TABLE 75.2 Federal and Professional Guidelines: Scope of Guideline Application

	NIH[1]	NAS	ISSCR
Research Using Previously Derived hESCs:			
Created using IVF for reproduction; no longer clinically necessary*			
Created by Somatic Cell Nuclear Transfer (SCNT)			
Created by parthenogenesis			
Created using IVF specifically for research purposes			
Generation and Derivation of new hES cell lines			
Use of hESCs or induced pluripotent stem cells to create chimeras:			
The introduction of hES or iPS cells into non-human primates at any stage of embryonic, fetal or postnatal development	[2]		
The introduction of hES or iPS cells into animals other than humans or primates at any stage of embryonic, fetal or postnatal development	[2]		
The introduction of hES or iPS cells into non-human primate blastocysts or of any embryonic stem cells into human blastocysts			
Allowing any animal to breed that has hES or iPS cells that could contribute to the germ line			
Research using SNCT for reproductive cloning			
Research involving human totipotent cells			
Research involving *in vitro* culture of any intact human embryo for longer than fourteen days			

* Eligible for federal funding.

hESCs, *Human embryonic stem cells;* iPScs, *induced pluripotent stem cells;* ISSCR, *International Society for Stem Cell Research;* IVF, *in vitro fertilization;* NAS, *National Academies of Science;* NIH, *National Institutes of Health.*

Key:

Green: Permitted after currently mandated reviews; all research under this designation must follow all accompanying guidelines pertaining to consent, reimbursement, banking, etc.

Yellow: Permitted only after additional review and approval.

Red: Not permitted by guidelines at this time. In the case of NIH, research pertaining to these areas is not eligible for federal funding. The other two guidelines are not legally binding, but institutions, states, and private funders are advised to consider guidelines when considering which projects to fund and approve.

Gray: Not considered by guidance.

Notes:

[a] *The NIH guidelines speak and apply to only that research which is federally funded. Thus areas of stem cell research that are not addressed in the NIH guidelines are neither eligible for federal funding nor controlled by the NIH's guidance.*

[b] *The NIH 2009 guidelines prohibited funding on the creation of human–animal chimeras in which human stem cells are introduced into nonhuman primate blastocysts. In 2015, the NIH created a moratorium on funding for the integration of human stem cells in all nonhuman vertebrate animals' pregastrulation-stage embryos. The NIH issued a call for comments in 2016 to consider ending the moratorium and consider extending the scope of chimera research. For more, see the "Animal–Human Chimeras" section at the end of this chapter.*

TABLE 75.3 Federal and Professional Guidelines: Research Guidelines

	NIH	NAS	ISSCR
INFORMED CONSENT			
Donors must understand all options for embryos no longer needed for reproductive purposes	x		
Decisions related to creation of human embryos for reproductive purposes must have been made free from influence of researchers proposing to derive or utilize hESCs in research	x	x	x
Donors must provide consent for embryo donation to research at the time of donation	x	x	x
Donors must provide consent for tissue and cell donation if at any time these materials are to be used for research involving the creation of human embryos			x
All gamete donors must provide consent for embryo donation		x	x
During the consent process, donors must be informed: **1.** That embryos would be used to derive hESCs and may be kept for many years **2.** What would happen to embryos in the derivation of stem cells for research **3.** That the research is not intended to provide direct medical benefit to the donor **4.** That the results of research may have commercial potential, and the donor(s) would not receive financial or other benefits **5.** Whether information that could identify the donor(s) would be available to researchers	x	x	

Continued

TABLE 75.3 Federal and Professional Guidelines: Research Guidelines—cont'd

	NIH	NAS	ISSCR
During the consent process, donors must be given: **1.** The information that hESCs or iPSCs may be used for research on transplantation, genetic manipulation, or the mixing of human and nonhuman cells in animal models **2.** A summary of the risks involved to the donor **3.** A statement as to whether donors wish to be contacted in the future to receive information obtained through studies of the cell lines, if identities are to be retained		x	
Donors must be informed that they can withdraw consent at any time until stem cells are derived	x		
Donors must be informed of institutional policies on return of incidental findings			x
REIMBURSEMENT AND PAYMENT			
No payments, cash or in kind, can be offered for donated embryos	x	x	x
Research oversight bodies must authorize proposals to reimburse or compensate donors			x
Compensation for egg donors should cover "direct costs" of time, effort, and inconvenience only		x	x
OVERSIGHT			
Mechanisms should exist on institutional, regional, and/or national level(s) to oversee and approve novel technologies, methods, or cell types		x	x
IRBs should review the procurement of all gametes, morulae, blastocysts, or somatic cells for the purpose of generating new hESc or iPSc lines		x	
IRBs should approve any processes pertaining to banking of hESc lines		x	
DERIVATION, BANKING, AND DISTRIBUTION			
Institutions engaged in obtaining and storing hESc lines should develop standards for: **1.** Committee review and oversight of banking and withdrawals **2.** Documentation requirements (donor consent and IRB approval forms, medical, clinical, and diagnostic information, infectious disease screening, culture conditions, and cell line characterization) **3.** A secure system for privacy of donors, especially with identifiable information		x	
National and international repositories should accept deposits of newly derived stem cell lines and distribute them on an international scale			x

hESc, Human embryonic stem cell; *iPSc*, induced pluripotent stem cell; *IRB*, Institutional Review Board; *ISSCR*, International Society for Stem Cell Research; *NAS*, US National Academies of Science; *NIH*, National Institutes of Health.

or licensing authority. Substantively, bans on creating embryos by cloning are common, although not universal, as are bans on creating embryos by fertilization except as part of fertility treatment, which means that research in many, although certainly not all, nations is limited to surplus or spare embryos. The US approach of regulating federally funded research but leaving nonfederally funded research unregulated is highly unusual by international standards [65].

In the United Kingdom, comprehensive legislation governing all research and medical use of human gametes and human embryos has existed since 1990 [66]. A major feature of the legislation is that it institutes few bans, but requires that all collection and use of embryos and gametes be licensed and overseen by an independent body called the Human Fertilisation and Embryology Authority (HFEA). The existence of this legislation and the HFEA before hESCs were first isolated meant that the research already had a regulatory system into which it could immediately be slotted, obviating the need for significant new legislative action by the UK government in response to the research (although regulations were promulgated in 2001 to add three new purposes for which research on embryos is permitted, including increasing knowledge about serious disease) [67]. Some scholars have urged the United States to adopt a similar regulatory system, under which very few activities are banned outright but instead require a license and are subject to oversight [68].

In neighboring Canada, national funding guidelines similar to the policy formulated by President Clinton were released in 2002 by the Canadian Institutes of Health Research (CIHR) [69]. Significant terms of these guidelines include that all research using CIHR funds is subject to national oversight by the Stem Cell Oversight Committee, that all embryonic stem cell lines generated using CIHR funding must be recorded in a national electronic registry and made available to other Canadian academic researchers at a cost, and that stem cells can only be extracted from

spare embryos (embryos cannot be created for research purposes, including by cloning). In 2014, the CIHR guidelines were integrated into the 2nd Edition of Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans in December 2014 [70].

The CIHR guidelines were followed in 2004 by the Assisted Human Reproduction Act, which, like the legislation in the United Kingdom, regulates much more than just embryonic stem cell research [71]. Several provisions of the Act, however, directly impact embryonic stem cell research. In particular, the Act prohibits creating a cloned embryo and creating an embryo for "any purpose other than creating a human being or improving or providing instruction in assisted reproduction procedures," thereby limiting embryonic stem cell research in Canada to embryos originally created in the course of fertility treatment (although the legislation does not specify that such embryos be surplus to the fertility needs of the donors) [72].

The distinction that many policies draw between stem cell research involving embryos created for use in research (including by cloning) and research involving embryos originally created for use in fertility treatment also appears in Australia's Research Involving Human Embryo's Act 2002 and the Prohibition of Human Cloning Act 2002. Both Acts limit research to "excess ART embryos," defined as embryos created for use in the assisted reproductive technology treatment that are now excess to the needs of the woman or couple for whom they were created [73]. The Prohibition of Human Cloning for Reproduction and the Regulation of Human Embryo Research Amendment Bill 2006 retained these conditions, but further specified laws on cloning, allowing for SCNT while prohibiting reproductive cloning [74]. Similar conditions are attached to research in other nations, including France, Denmark, Finland, Greece, the Netherlands, and Japan (a full breakdown of country policies can be seen in Table 75.4).

South Korea, a country active in stem cell research, is in the unusual position of permitting the creation of cloned embryos for research into rare or incurable diseases, but prohibiting the creation of embryos for research by IVF. Under a 2004 law, revised in 2008, IVF may only be used to create embryos for reproductive purposes, which can later be donated to research if not used. Cloned embryos may only be created for use in research into the treatment of rare or incurable diseases [75]. In early 2006, cloning research led by Woo-Suk Hwang of Seoul National University was found to have included a number of questionable practices, including that some egg donors were members of the research team, some egg donors were paid to donate, and researchers accompanied some egg donors as they underwent the extraction procedure [76]. Following charges of embezzlement and falsifying results against the prominent stem cell researcher, the South Korean government halted approvals and funding for stem cell research until early 2009. Embryonic stem cell projects have been approved again, and since 2012 South Korea has reemerged as a leader in stem cell research, due both to a federal funding boost and considerable regulatory freedom [77]. In Israel, another country active in stem cell research, no legislation regulates the field, although there is a law against implanting a cloned embryo [78]. Therefore derivation of stem cells from embryos created for research use, including by cloning, is allowed (although to date there are no reports of Israeli scientists creating cloned embryos) [79]. Under Singapore's 2004 legislation, research is permitted on spare embryos and embryos created for research, including by cloning [80]. With over 40 active stem cell research groups in the country limited by very few regulations, the country has been called "Asia's Stem Cell Center." In 2012, Singapore established a Bioethics Advisory Committee to oversee research, but provides very few legal or regulatory barriers [81].

SELECTED ETHICAL, LEGAL, SOCIAL, AND POLICY QUESTIONS OF STEM CELL RESEARCH

As embryonic stem cell research moves forward in the United States, various ethical and policy questions arise, some of which have already been discussed. For example, should researchers create embryos in the laboratory by fertilization or cloning or should they only use spare embryos? Either way, they will need to interact with fertility clinics or with egg, sperm, or embryo donors, raising questions about how those interactions should be conducted. Should researches pay fertility clinics for procuring gametes and embryos for stem cell research? How should gamete and embryo donors be approached to donate, precisely whose consent should be required, and should the donors be compensated? Once researchers extract cell lines, are they obliged to make those lines available to other researchers? Should researchers patent new cell lines or new stem cell-related processes? If they do obtain patents, what practices should they follow in licensing those lines or processes? Should researchers be allowed to mix human cells and animal cells in the creation of chimeras or hybrids, and what could happen if they do so? Here we address several of these issues in some detail.

TABLE 75.4 Human Embryonic Stem Cell Research Laws and Funding in Countries with Applicable Policies

Country	hESc Research Legal	Sources of Stem Cells			Cloning (SCNT) Legal	
		Imported hESC Lines	Excess IVF Embryos	Embryos Created for Research (IVF or, if permitted SCNT)	Therapeutic / Research	Reproductive
Argentina						
Australia						
Austria	~	*a*				
Belgium						
Brazil						
Bulgaria	*					
Canada						
Chile	*b*					
China						
Columbia						
Costa Rica						
Croatia						
Cyprus						
Czech Republic						
Denmark						
Ecuador						
Estonia	*					
Finland				+		
France						
Germany	+	*c*				
Greece						
Hungary						
Iceland						
India				^		
Iran	*d*					
Ireland				*e*		
Israel						
Italy	+					
Japan						
Latvia						
Lithuania	*f*					
Mexico	*g*					
The Netherlands						
New Zealand			*h*			
Norway						
Poland	~					
Portugal						
Romania	*i*					
Russia						
Singapore	*j*					
Slovakia	+					
Slovenia						
South Africa						
South Korea					*k*	
Spain						
Sweden					*l*	
Switzerland			^			
Trinidad and Tobago						
Tunisia						
Turkey	*m*					
United Kingdom						
United States			*n*		*n*	

hESc, Human embryonic stem cell; *IVF*, in vitro fertilization.

Key:

Green: Permitted by Law or Guidance (some uses may be limited by rules or regulations)

Red: Prohibited by Law (some uses may be exceptions, and allowed under specific conditions)

Grey: Not considered in law — implicitly permissible, not governed by regulations or guidance

TABLE 75.4 Human Embryonic Stem Cell Research Laws and Funding in Countries With Applicable Policies—cont'd

** The country doesn't explicitly regulate stem cells, but one of the country's laws allows surplus IVF embryos to be used for research purposes, suggesting that hESC research is implicitly permissible.*
+ Banned unless medically, scientifically, and therapeutically vital, in particular for the specific embryo involved.
~ The country has no legislation on stem cell research, but the creation of embryonic stem cell lines and research on such lines is de facto *prohibited.*
^Researchers must provide explicit justification for research or use of embryos/ESCs and establish that creation / use of embryos is essential for research.
Country Specific Notes:
[a] In Austria, ESC research is de facto *prohibited because embryos and gametes cannot be donated for purposes other than assisted reproduction with heterosexual couples. However, the use of imported hEScs was not addressed explicitly by law and thus is implicitly permissible. While the derivation of embryonic stem cells from human embryos is currently prohibited, the Austrian Bioethics Commission has advocated for hESC derivation of surplus embryos.*
[b] The law in Chile is unspecific. A 2006 law defends the "protection of human life from the moment of conception," and states that no human embryos can be destroyed to obtain stem cells, and that ES cell lines only can be used for "therapeutic diagnosis or scientific research".
[c] The creation of and research with hES cell lines is banned in Germany as a "matter of principle." In 2008, Germany eliminated a past law that made it a criminal offence to use hES cells in research conducted outside German's borders. Research on imported hES cell lines is allowed, but hES cells cannot be derived in Germany, and must have been imported before May 1, 2007.
[d] ESC research has been permitted in Iran since 2002, when Supreme Leader Ayatollah Khamenei issued a 'stem cell fatwa*' that declared that human embryo research was consistent with Shia tradition. Iranian scientists and bioethicists have since developed ethics guidelines for hES cell research.*
[e] Ireland has no legislation on embryo or stem cell research, but the Irish Medical Council has banned medical practitioners from creating embryos specifically for research.
[f] Lithuania limits hESC and embryo research to non-interventional clinical observations only.
[g] Mexico is currently debating an amendment that would ban all research on human embryos, thereby halting the creation of and any research on hESC lines.
[h] In New Zealand, research can only be conducted on non-viable embryos (in contrast with viable excess IVF embryos, permitted by other countries).
[i] Stem cell research is only allowed in Romania under official approvals. There is no regulation of IVF, research on embryos, or hESCs.
[j] Embryonic stem cell research is permitted in Singapore under recommendations from the Bioethics Advisory Committee, which are adhered to by the scientific community. The creation of embryos through SCNT is permitted on a case-by-case basis to derive patient-specific cells.
[k] South Korea permits SCNT as a source for hESCs (in addition to excess IVF embryos) despite the ban on production of non-reproduction embryos; SCNT can be used "for the purpose of conducting research aimed at curing rare or currently incurable diseases."
[l] Sweden permits therapeutic cloning if hereditary genetic traits remain unchanged.
[m] Turkey allows for non-embryonic stem cell research when officially approved, no hESC research is permitted.
[n] United States law and guidelines allow for SCNT and the derivation of hESCs at large, but neither are permitted as part of federally funded research. Some individual US states have laws addressing SCNT and hESC research.
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Compensating Egg Donors

The South Korean stem cell controversy over the procuring of eggs in South Korea for use in cloning research brought additional attention to egg donation. Findings related to the donation process raised concerns about whether the women who donated eggs to Hwang's research did so completely voluntarily. Voluntariness is a core commitment of modern research ethics [82], which generally translates into requirements that no one is pressured to participate in research and that each participant is able to withdraw from the research at any time without endangering ongoing medical care [82]. Researchers usually avoid enrolling family members and employees because they might reasonably feel significant pressure to participate. The commitment to voluntary participation, and specifically the derived right to withdraw from the research at any time, could also be under threat if researchers physically accompany volunteers through procedures as Hwang and his colleagues apparently did.

Concern to protect voluntariness is also a major factor motivating bans on compensating egg donors, the argument being that the need for money could compel participation, especially, though not exclusively, among the poor. Nevertheless, it is unclear whether the concern is sufficient to justify a complete ban on compensation, particularly in a US context. As the National Academies committee noted, many other research participants in the United States receive compensation in exchange for their involvement in research [57]. Such payment, particularly where modest, is said to be not only a necessary incentive, but also fair treatment of research subjects (after all, the researchers and their staff will be paid for the time and resources they contribute toward the research). The concern about voluntariness in the hESc context is likely heightened because egg donation is time-consuming, painful, and involves some risk. Egg donors are injected with drugs over weeks so that they superovulate (produce many eggs). The eggs are then removed from the woman by either inserting a hollow needle through her vagina or by laparoscopic surgery. Risks of the stimulation and egg-collection process include hot flashes, headaches, sleeplessness, mood alteration, ovarian hyperstimulation syndrome, nausea, vomiting, pain, bleeding, and infection. There is even a controversy over a possible danger of ovarian cancer from the medications and ovarian stimulation for IVF [83,84]. Could payment encourage some women to donate eggs even though donation might be painful and pose a risk to their health? The answer is yes. However, whether the risk and pain are unacceptable is another issue. In theory at least, if an IRB approves research involving egg donation, it has decided that the risk to donors is reasonable in relation to the importance of the knowledge that may reasonably be expected to result [85]. Compensation is not supposed to be offered to research participants to seduce them to take an unacceptable risk.

But voluntariness may not be the only concern about paying egg donors in stem cell research. There is also some opposition to paying anyone for providing bodily materials (rather than solely for their time and effort), for example, as expressed in a federal law prohibiting payment for organ donation (although that law expressly does not apply to blood, sperm, or human eggs) [86]. The stance against the sale of bodily materials is well defended in scholarly circles. Bioethicist Thomas Murray argued nearly 30 years ago that all donations of body parts, whether for research or for clinical treatment, should be gifts and not sales [87]. Others, however, including law professor Lori Andrews, writing around the same time, countered that individuals "have the autonomy to treat their own (body) parts as property," particularly those parts of the body that they can regenerate [88].

The debate about compensating egg, sperm, and embryo donors continues today. Beyond clarifying their definition of "direct reimbursable expenses" as "costs associated with travel, housing, child care, medical care, health insurance, and actual lost wages," the NAS guidelines included a note on autonomous choice:

> To facilitate autonomous choice, decisions related to the creation of embryos for infertility treatment should be free of the influence of investigators who propose to derive or use hES cells in research. Whenever it is practicable, the attending physician responsible for the infertility treatment and the investigator deriving or proposing to use hES cells should not be the same person.

Although included in the final iteration of the guidelines, the recommendations leave the issue open for interpretation by researchers, and further questioning by the scientific and policy communities at large [58]. The only state that provides for compensation of egg donors is New York, which in 2009 announced that its funds could be used to offer women up to $10,000 for donation of eggs to hESc research.[1]

Commercialization and Access to Treatments

Another argument against paid donation is that it adds to the costs of conducting research and thereby the price of eventual treatments. But this argument works best if the same spirit is adopted by the scientists, institutions, and companies involved in the research and in any eventual treatment. Indeed, a commitment to scientific progress and widely available treatments in stem cell research might entail a commitment by all those involved to, for example, banking and widely distributing new cell lines, participating in international collaboration, and adopting patenting and licensing practices designed to facilitate access (including possibly not patenting some discoveries at all) [89]. These concerns about secrecy, privacy, access, and commercialization in stem cell research mirror a larger debate in biomedical research in general [90].

[1] The legal status of compensation limits like that announced by New York's Empire State Stem Cell Board is currently unclear in light of a recent class action lawsuit filed by egg donors in opposition to fertility industry caps on payments (*Kamakahi et al. v. American Society for Reproductive Medicine et al.*, Case No. 3:11-cv-01781-JCS, in the US District Court for the Northern District of California).

In terms of access, particular emphasis is often put on patenting and licensing practices, which can have an enormous impact on progress made by other researchers, as well as on the availability of eventual treatments [91]. Indeed, a number of patents already attach to embryonic stem cell research [92], including a long-standing patent claiming a purified preparation of primate (including human) embryonic stem cells and a method for isolating them [93]. To establish its 2001 registry, the NIH negotiated with patent holders to issue licenses to noncommercial entities royalty free so that research could move forward. The NIH-negotiated licenses, however, explicitly cover only research and do not extend to commercial activities [94]. It is not yet known whether these same terms apply to research using lines in the new NIH Human Embryonic Stem Cell Registry.

As a general matter, patents in biomedical research can restrict other researchers from using a specific method or material, creating monopoly-like conditions, and hampering the progress of research. These conditions could significantly raise prices and reduce equitable access to treatments. Access for research and treatment was among the issues motivating the lawsuit over gene patents held by Myriad Genetics that was resolved in the Supreme Court in 2013. While the reasoning in the *Myriad* case refers specifically to the ineligibility of DNA sequences for patents, some have predicted that the case's vague precedent for "natural" biologic materials could impact stem cell patents [95,96]. If it does not, the patentability of stem cell research methods and future treatments could raise treatment prices and create significant disparities in treatment access. At the time of writing, the future of the Affordable Care Act and other insurance and drug pricing regulations is uncertain in the United States. Without suitable insurance and treatment cost protections, disparities in access to stem cell treatments may be even more pronounced.

Patenting and licensing issues were to some extent anticipated in California's Proposition 71, which included a provision requiring the establishment of standards in all grants and loans allowing the state to financially benefit from licenses, patents, and royalties and resulting from the research activities funded under the measure [55]. Regulations implementing these standards require that a portion of profits be returned to the state [97].

Animal–Human Chimeras

To develop and test possible human clinical uses, human stem cells are being implanted into nonhuman animals at various stages of development. "Human–animal chimeras" are created by introducing human genetic material into nonhuman animal embryos. Human stem cells can be inserted into animal embryos, fetuses or postnatally, and will grow and develop into various organs and systems within the animal. The ethical and legal questions surrounding chimera animals depend on where and when human stem cells are introduced into the animal system: is the animal a primate or vertebrate that is genetically similar to humans; are stem cells inserted into the blastocyst, embryo, or later stage in the animal's development; will the human cells impact an animal's nervous system or enter its germ line for reproduction; and will the animal be brought to term or allowed to reproduce?

Although guidelines vary as to how they address the creation of animal–human chimera (refer to Tables 75.2 and 75.3), they are all attentive to several similar factors. First, whether or not the animal into which the human cells are introduced is a close genetic relative of humans is considered to be important both clinically and ethically. From the perspective of clinical validity, a primate or other vertebrate animal is a more useful test model than an animal with dramatically different genetic and biological structures from humans. However, from an ethical perspective, some commentators have raised concerns about the "humanization" of animals. If an animal species is a close genetic neighbor to humans, it is thought that human stem cells introduced early in development could be sufficiently integrated into the animal in ways that could be "morally humanizing"—that is, that move the animal's moral status toward human beings thereby creating a novel creature with a novel, and unclear, moral status [98].

This concern about moral status is also behind two other important issues about animal–human chimeras: the location and number of the human cells within the nonhuman animal, and the stage of development during which stem cells are introduced in animals. If stem cells are introduced in a postnatal animal, they are unlikely to become integrated into systems beyond the organ or tissues at which they were targeted [98]. However, if human stem cells are transplanted into animal embryos (single celled) or blastocysts (between 50 and 100 cells), they are considerably more likely to become integrated into many if not all of the animal's biological systems, including the animal's central nervous or reproductive systems. Could human stem cells in the brain of a pig, for instance, "humanize" it [98]? Could human cells enter the animal's reproductive system causing it to produce human sperm or eggs? In response to the latter concern, prohibition on breeding of any animals into which human stem cells have been transplanted is widespread [44,54,64].

The NIH 2009 guidelines specifically stated that research introducing embryonic or iPScs into any animals at any stage of development could not be covered by federal funding [44]. In 2015, the NIH extended the federal funding ban on chimera research, announcing a moratorium on funding for research introducing human pluripotent cells into all nonhuman vertebrate animal pregastrulation embryos [99]. After a public comment and workshop period, NIH released a call for public comments in September 2016 [100] on a proposal to expand the prohibition on introducing hEScs or iPScs into nonhuman primate embryos to include preblastocyst-stage in addition, to blastocyst-stage embryos. Within its proposed changes, the NIH proposed to potentially permit (as decided by the steering committee) funding for research integrating human stem cells into nonhuman vertebrate embryos up through the end of the gastrulation stage (lifting the 2015 moratorium), as well as research in which human stem cells are introduced into postgastrulation, nonhuman, nonrodent mammals, which may present a "substantial functional modification to the animal brain by human cells" [98]. This final provision, if supported by federal funding, could result in researchers being able to create animals that present the aforementioned risk of "humanization." Such a concern has raised several questions. For instance, what addition of human materials, if anything, would constitute an "elevation of moral status" in a research animal such that it could no longer be used for research? Furthermore, a concern about humanization begs a more fundamental question about our own humanness, moral status, and consciousness. Some have argued that the interactions and impact of human material in chimeras will not reach a point at which these questions truly become necessary, and that instead, issues surrounding animal welfare and the potential harms that we may have on research animals are more important at present [98] (Currently, many research centers mandate that Institutional Animal Care and Use Committees oversee animal–human chimera research and other stem cell transplants occurring in nonhuman animals.) Nonetheless, as stem cell technologies and research move forward, questions about research that blurs the line between humans and nonhuman animals will likely remain at the forefront of ethical and policy decision-making.

CONCLUSION

As this brief overview shows, stem cell research, and in particular hESc research, has led to the development of numerous policies. In the United States, as well as in some other nations, these policies have responded to the controversial nature of research involving the destruction of human embryos. The US federal funding policy of 2001, which limited the use of federal funds in embryonic stem cell research, generated a lot of attention, including significant criticism. In response to the 2001 policy's limits, funds for embryonic stem cell research were provided by private donors, charitable organizations, and some states. In 2009, President Obama repealed the 2001 policy. Nevertheless, federal funding, while now more widely available, is still limited to research on cells derived from spare embryos and cannot be used to derive new cell lines.

As research on embryonic stem cells taken from a variety of sources moves forward in the United States and internationally, some of it without funding or guidelines, a range of policies are developing that respond to a number of important issues, including local and national oversight, the role of donors in the research, the consequences of commercial interests, the creation of animal–human chimeras, and the extension of stem cell research technology using new technologies such as gene editing. At the time of writing, it is still unclear if and how the Trump administration will approach the ethical issues associated with embryonic stem cell research and funding. Simultaneously, stem cell and greater bioscience technologies are developing into increasingly complex arenas, including involving advances in gene editing. It will be important to continue to observe how stem cell research remains intertwined with ethical, legal, and policy questions on domestic and international fronts.

List of Acronyms and Abbreviations

CIHR Canadian Institutes of Health Research
CIRM California Institute for Regenerative Medicine
hESc Human embryonic stem cell
HFEA Human Fertilisation and Embryology Authority (United Kingdom)
HHS Department of Health and Human Services, once was DHEW: Department of Health, Education and Welfare
iPSc Induced pluripotent stem cell
IRB Institutional Review Board
ISSCR International Society for Stem Cell Research

IVF In vitro fertilization
NAS National Academy of Science
NIH National Institutes of Health
SCNT Somatic cell nuclear transfer

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CHAPTER

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Ethical Considerations*

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INTRODUCTION

Since the first development of human embryonic stem cells (hESCs) in 1998 [2,3] there has been a consensus in the scientific community that pluripotent cells hold great promise for developing new treatments for a variety of serious and currently untreatable disease conditions [4,5]. Although clinical successes in this area have been slow in coming, research is ongoing and novel therapies are beginning to show promise in clinical trials [6,7].

Nevertheless, because hESC research involves the manipulation and destruction of human embryos, the field has also been a focus of ethical controversy and opposition. In the course of these debates, many challenging ethical questions have been raised. Scientists, clinicians, or patients using hESCs or therapies must formulate their own answers to these questions. Society, too, must address them to determine the extent to which hESC research may require oversight and regulation. This chapter presents the most pressing of these questions, and critically examines some of the answers that have been proposed to them.

IS IT NECESSARY TO USE HUMAN EMBRYOS?

Whether it is permissible to destroy human embryos to create hESC lines is a leading question, to which we turn in question 2 later. However, in late 2007, teams led by Yamanaka in Japan and Thomson in the United States announced success in the use of gene transfer technology to produce induced pluripotent stem cell (iPSC) lines [8,9]. Some have argued that this development obviates the need to develop or use hESC lines [10]. They assume that if we can directly manipulate somatic cells and perhaps even produce patient-specific (autologous) stem cells there is no reason to destroy human embryos or use hESCs derived from such destruction. However, there are both scientific and ethical reasons for questioning this assumption.

Scientifically, there is a question of whether iPSCs will prove suitable for use in human transplant and cell regeneration therapies. Early iPSCs exhibited high rates of tumorigenicity in mice, possibly a result of the use of retroviral vectors to carry pluripotency-inducing transcription factors, including the cancer-related factor c-Myc [11,12]. Reports suggest that the genomic reprogramming process in iPSCs may be less complete than that which takes place in the fertilized egg, causing the resulting cells to exhibit abnormal expansion and early senescence [13]. There is also a concern that because iPSCs are produced from somatic cells that have been exposed to aging and toxins, they may exhibit harmful mutations when used in clinical therapies. Because of these concerns and the possible costs of patient-specific therapies, some argue that banks of tissue-matched hESCs offer better prospects for therapies. In a review of these issues, Hug and Hermerén conclude that "if we consider all the aspects of safety, it is hardly possible to determine which therapy based on which type of cells would be safer according to the present state of knowledge" [14].

Research to answer these questions is ongoing (for example, [15,16]), but doing so will require a better understanding of the reprogramming process in embryonic cells. For this reason alone, the use of human embryos is likely

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to continue to be an important feature of stem cell research [17]. At this time, there exist many useful hESC lines created from donated human embryos. There are also currently hundreds of thousands of frozen embryos remaining from infertility procedures that will likely be destroyed and that could be used for research [18]. If stem cell research involving embryos were halted, this vast resource would go to waste. Many people feel that it is unwise to foreclose any of the available paths to developing pluripotent cells for regenerative medicine research. The view of the National Research Council remains in force: "The application of stem cell research to therapies for human disease will require much more knowledge about the biological properties of all types of stem cells" [4].

Ethically, it can also be asked whether the production and use of iPSCs is free of concerns. It is widely assumed that iPSCs avoid ethical controversy because they are produced from somatic cells, which unlike early embryos have no inherent potentiality of developing into a human being. But the work of Nagy and others shows that embryonic stem cells, when inserted into tetraploid embryos, are able to develop the placental material needed for further development [19–24]. This possibility raises complex ethical questions. Is potentiality morally relevant if it is accompanied by such intensive technical interventions? Embryos produced by in vitro fertilization (IVF) or nuclear transfer (cloning) also require intensive technical interventions, and opponents of hESC research normally condemn the use of these as sources for hESCs. If it is acknowledged that the potentiality for full human development, however assisted, confers moral status on an entity, then iPSC research may not be the ethical panacea that many of its proponents believe it to be. As Giuseppe Testa [25] observes:

> The point is not that one can no longer use the potentiality argument because fibroblasts might now be considered as potential persons in a more concrete way than ever before… That is, there is no longer, if there ever was, any ready-made grid of boundaries that biology can let us see as if they were simply out there and that can serve as neutral justification for political choices.

Finally, it might be asked whether, even within the framework of hESC research, we must destroy embryos. In 2005, the Bush administration's President's Council on Bioethics issued a White Paper encouraging research in alternate methods of hESC derivation, including the use of arrested or developmentally nonviable embryos [26]. Several of these proposals, such as the deliberate genetic manipulation of embryos to prevent their normal development, are not free of ethical controversy [27,28]. A method involving single-cell blastomere biopsy has also been developed and implemented that could obviate the need to destroy the embryo to develop an hESC line [29]. While this approach raises questions of risk to the manipulated embryos, it can be justified in the context of preimplantation genetic diagnosis (PGD), where cells are routinely removed as a diagnostic procedure.

Reviewing all these considerations, we can say that considered scientifically and ethically there is no easy route around the destruction and use of human embryos in pluripotent stem cell research.

IS IT MORALLY PERMISSIBLE TO DESTROY A HUMAN EMBRYO?

hESC lines are usually made by chemically and physically disaggregating an early, blastocyst-stage embryo, and removing its inner cell mass. At this stage the embryo is composed of approximately 200 cells, including an outer layer of differentiated placental material, and the undifferentiated (pluripotent) cells of the inner cell mass. The embryo dies as a result of this procedure. New methods that permit the development of pluripotent cell lines without destroying an embryo have not yet replaced this standard method for developing hESCs, and most existing hESC lines were created this way. Hence the question remains: may we intentionally kill a developing human being to expand scientific knowledge and potentially provide medical benefits?

At one end of the spectrum of answers are those who believe that, in moral terms, human life begins at conception when a new, self-developing genome comes into being. For many of those holding this view, the early embryo is not morally different from a child or an adult human being. It cannot be used in research that is not to its benefit, and it cannot be used without its consent [30–32]. Furthermore, proxy consent by parents in such cases is inadmissible, since it is an accepted rule of pediatric research that parents may not volunteer a child for risky studies that are not to its benefit. Many Roman Catholics, evangelical Protestants, and some Orthodox Jews take the position that life (morally) begins at conception, and they oppose hESC research.

At the other end of the spectrum are those who believe that the embryo is not yet fully a human being in a moral sense. They hold a "developmental" or "gradualist" view of life's beginning [33–37]. They do not deny that the early embryo is alive and has the biological potential to become a person, but they believe that other features are needed for the full and equal protection we normally accord children and adults and that these features only develop gradually across the full term of gestation. These features include such things as bodily form and the ability to feel or

think. Since the early embryo lacks organs, it cannot have these features or abilities. They also note that the very early embryo lacks human individuality, since it can still undergo twinning at this early stage, and two separate embryos with distinct genomes can fuse to become a single individual [38]. Some dismiss this argument, maintaining that the possibility of twinning or fusion does not reduce the genetic uniqueness or moral status of the earliest developing cells [39], but it is hard to see how the claim that "a person begins at conception" can easily withstand these biological facts. Finally, the very high mortality rate of such embryos (most never implant) reduces the force of the argument from potentiality [40,41]. Those who hold this developmental view do not agree on the classes of research that warrant the destruction of embryos, but most support some form of hESC research. Their reasoning is that, although the early embryo merits some respect as a nascent form of human life, the lives and health of children and adults outweigh whatever claim it possesses [42].

Each individual faced with involvement in hESC research must arrive at his or her own answer to the question of the status of the early human embryo and when, if ever, it may be destroyed. Legislators and others must also wrestle with these issues. On March 9, 2009, President Barack Obama issued an executive order authorizing federal funding for research involving the use, but not the derivation, of hESC lines. Whether this position will be sustained by the Trump administration remains to be seen.

MAY ONE BENEFIT FROM OTHERS' DESTRUCTION OF EMBRYOS?

If, as some maintain, the human embryo is a morally protectable entity that cannot be intentionally destroyed, can researchers, clinicians, or patients justify the downstream use of a cell line produced from its destruction? This raises the more basic question of whether we can ever benefit from deeds with which we morally disagree or regard as wrong [43,44]. It is also the question of when a connection with wrongdoing becomes complicit with it [45].

Why is it morally wrong to benefit from others' misconduct? One answer is that by doing so we may encourage similar deeds in the future. This is most apparent in cases where our conduct directly instigates wrongdoing, such as when we authorize theft or receive stolen goods [45]. However, it seems less objectionable to benefit from others' wrongdoing when their deeds are independently undertaken and not in any way prompted or encouraged by us. For example, few would object to using the organs from a young victim of a gang killing to save the life of another dying child. The use of organs benefits one person and in no way encourages teen violence. Can similar logic apply to stem cell research using spare embryos remaining from infertility procedures?

It helps to remember that most embryos used to produce hESC lines are left over from infertility procedures. Couples using IVF routinely create more embryos than can safely be implanted. There are already hundreds of thousands of these embryos in cryogenic freezers in the United States and around the world [18]. Despite strenuous efforts, including some supported by the US government, few frozen embryos are adopted [46,47]. Most of these supernumerary embryos will be destroyed. In 1996, British law mandated the destruction of 3600 such embryos [48]. Does using hESC lines made from surplus embryos encourage either the creation or destruction of embryos?

Although a downstream researcher, clinician, or patient may abhor the deeds that led to the existence of an hESC line, including the creation and destruction of excess human embryos in infertility medicine, nothing that a recipient of an hESC line chooses to do is likely to alter, prevent, or discourage the continuing creation or destruction of human embryos, or make the existing lines go away. Those who use such embryos may also believe that if they refuse to use an hESC line, they would forgo great therapeutic benefit. People in this position will struggle with the question of whether it is worthwhile to uphold a moral ideal or engage in a symbolic act when doing so has no practical effect, and when it threatens to expose others to harm.

It is noteworthy that in an August 2001 address to the nation, President George W. Bush adopted a version of the position that allows one to benefit from acts one morally opposes. Stating his belief that it is morally wrong to kill a human embryo for others' benefit, the President nevertheless permitted the use of existing stem cell lines on the grounds that the deaths of the embryos had already occurred [49]. Similarly, Germany permits the importing and use of hESC lines created before January 1, 2002, the date on which the Bundestag passed a law governing such matters (this date was later moved to May 2007). These initiatives reflect the belief that it does not encourage further destruction of embryos to permit the use of previously generated cell lines. President Bush did not go so far as to permit the use of lines that could in the future be derived from embryos certainly slated for destruction because their progenitors choose never to use them. However, in July 2009, the National Institutes of Health (NIH) implemented an executive order issued in March 2009 by President Obama that authorized funding for the use, but not the derivation, of new hESC lines from embryos remaining from infertility procedures [50]. Opponents of hESC research have criticized this derivation-versus-use distinction as morally problematical, but apart from the moral issues,

this distinction is legally necessary because the Dickey–Wicker Amendment prohibits federal funding for research in which a human embryo is destroyed [51].

In summary, the downstream use of stem cells derived from embryos remains a source of moral controversy and disagreement. Each researcher or clinician who opposes embryo destruction will have to examine his or her own conscience in the light of the foregoing considerations to determine how much they wish to associate themselves with the use of hESC lines created in ways to which they morally object.

MAY WE CREATE AN EMBRYO TO DESTROY IT?

Is it ever morally permissible to create an embryo deliberately to produce a stem cell line? This was done in the summer of 2002 at the Jones Institute in Norfolk, Virginia [52]. Those in favor of this research defend it on two grounds. First, they say that in the future, if we seek to develop stem cell lines with special properties, such as closer genetic matches to tissue recipients or properties introduced by gene editing (see question 7 later), it may be necessary to produce stem cell lines to order using donor sperm and eggs. Second, they argue that it is ethically better to use an hESC line created from embryos that have been produced for just this purpose, with the full and informed consent of their donor progenitors, than to use cell lines from embryos originally created for a reproductive purpose.

Those who believe the early embryo is our moral equal oppose the deliberate creation of embryos for research or clinical use. They are joined by some who do not share this view of the embryo's status, but who believe that it is morally repellant to deliberately create a potential human being only to destroy it [53]. They argue that this research opens the way to the "instrumentalization" of all human life and the use of children or adult human beings as commodities. Some ask whether such research does not violate the Kantian principle that we should never use others as "a means only" [54].

On the other side of this debate are those who believe that the lesser moral status of the early embryo permits its creation and destruction for lifesaving research and therapies [55,56]. The proponents of this research direction ask why it is morally permissible to create supernumerary embryos in IVF procedures to help couples have children, but morally wrong to do the same thing to save a child's life. They are not persuaded by the reply that the status of the embryo is affected by its progenitors' intent, and that it is therefore permissible to create excess embryos for a "good" (reproductive) purpose, but not for a "bad" (research) purpose. They point out that the embryo is the same entity, and its status should not depend on its progenitors' intentions. We do not ordinarily believe that a child's rights are dependent on its parents' intent or degree of concern [57]. They conclude that it is not parental intent that warrants the creation of excess of embryos in such cases, but the embryo's lesser moral status and the likelihood of significant human benefit from its use. These same considerations, they believe, justify deliberately creating embryos for stem cell research.

MAY WE CLONE HUMAN EMBRYOS?

This question arises in connection with the patient-specific stem cell technology known as "human therapeutic cloning." It involves the deliberate creation of an embryo using somatic cell nuclear transfer (cloning) technology to produce an immunologically compatible (isogenic) hESC line [58].

Immune rejection could occur if the embryo used to prepare a line of hESCs for transplant does not share the same genome as the recipient. This would be the case whether the cell line was created from a spare embryo or from one made to order. Therapeutic cloning offers a way around this problem. In the case of a diabetic child, for example, the mother could donate an egg whose nucleus would then be removed. A cell would be taken from the child's body and its nucleus inserted into the egg cytoplasm. With stimulation, the reconstructed cell would divide, just like a fertilized egg. If the resulting embryo were transferred back to a womb, it could go on to birth and become a new individual—a clone of the child. But in therapeutic cloning, the blastocyst would be dissected and an hESC line prepared. Growth factors could be administered to induce the cells to become replacement pancreatic cells for the child. Because these cells contain the child's own DNA, and even the same maternal mitochondrial DNA, they would not be subject to rejection. Research has also shown that the small amount of alien DNA from the mitochondria of a donor egg would probably not provoke an immune response [59].

Although this is a promising technology, it raises a host of novel ethical questions. One is whether the embryonic organism produced in this way should be regarded as a "human embryo" in the accepted sense of that term [60]. Those who believe that "life begins at conception" tend to answer this question affirmatively, even though cloned

"embryos" are not the result of sexual fertilization [61]. They believe that it is no more permissible to create and destroy a cloned embryo than to do so with one produced by sexual fertilization. They base their view on the biological similarities between cloned and sexually produced embryos and on the argument that both have the potential to become a human being. However, as we have seen, if the embryo's status rests on its potential, some degree of potentiality attaches today to all bodily cells, which no one would argue should be withheld from use in biomedical research or therapy.

Therapeutic cloning implies the availability of human oocytes and raises the special question of ovulation induction. This is an invasive medical procedure, with both known and undetermined risks [62,63]. Not only must egg donors be informed of these risks, but steps also must be taken to preserve the voluntary nature of their consent. This includes avoiding financial incentives that create an "undue influence" on donors' judgment. It also includes preventing them from being pressured into producing excess eggs or embryos for research in return for discounts on infertility services [64].

Fears about coercion and the exploitation of poorer women or women of color have led some to oppose paid egg donation for research [65,66]. Several states, including California and Massachusetts, have passed laws prohibiting this practice. Nevertheless, experience has shown that women will not donate eggs for either reproductive or research purposes without adequate compensation [67]. In the face of these problems, New York State reversed the legal trend and approved payment for research egg donation.

Those who defend payment for oocytes point out that payment to research subjects involved in risky research is a common practice. They also ask why it is permissible to pay reproductive but not research egg donors [68]. The Ethics Committee of the American Society for Reproductive Medicine has supported payment for research egg donors. It conceptualizes this in terms of appropriate compensation for a donor's time, inconvenience, and discomfort, but not for the eggs themselves [69,70].

Finally, there is a moral question specific to cloning itself. The more scientists are able to perfect therapeutic cloning, the more likely it is that they will sharpen the skills needed to accomplish reproductive cloning, which aims at the birth of a cloned child. There is a broad consensus in the scientific and bioethics communities that, for the foreseeable future, cloning technology poses serious health risks to any child born as a result of it [71]. There are also serious, unresolved questions about the psychological welfare of such a child [72]. Finally, there is the possibility that embryos created for therapeutic cloning research might be diverted to reproductive cloning attempts. All these concerns raise the question: do we really want to develop cloning technology for the production of isogenic stem cells if doing so hastens the advent of reproductive cloning [73]?

The advent of iPSCs and the prospect of efficiently producing patient-specific stem cell lines have weakened the arguments for therapeutic cloning with its many associated controversies.

MAY WE USE HUMAN STEM CELLS TO CREATE CHIMERAS?

In stem cell research, human-to-animal chimera experiments involve the transfer of pluripotent or multipotent human stem cells into animals at embryonic, fetal, or postnatal stages of development to study stem cell behavior [74]. Some forms of chimera research are common and widely acceptable. For example, hESCs are routinely used to form teratomas in immunodeficient mice to assess stem cell quality and developmental potential. The creation of humanized antibody systems in mice is also central to cancer immunotherapy research.

Researchers have expressed interest in creating humanized organs or tissues in larger animals for disease modeling, drug testing, and perhaps eventual organ transplant [75]. Animal models with human cells in the brain can be used to study many human brain diseases, including Parkinson's, Alzheimer's, and schizophrenia, and may be useful models for testing new drugs [76]. However, these proposals raise many ethical questions because hESCs or human islet-derived precursor cells (hiPCs) inserted into an animal embryo prior to gastrulation can incorporate themselves into neural or reproductive tissues. Modifying neural tissues risks the creation of an animal with significantly increased potential for human sentience and self-awareness, a concern that is greatly increased in the case of nonhuman primates whose brain architecture might reasonably support human-like cognition and feelings. Humanizing reproductive tissues risks the inadvertent mating of two animals with such tissue in their gonads and a resulting pregnancy or birth of a human child from an animal womb. Some have argued that either prospect is ethically unacceptable because it represents a threat to human dignity [77,78], although what constitutes human dignity or its violation is difficult to assess [74]. Nevertheless, in terms of recognized human subject protections and animal welfare considerations, few would disagree that it is wrong to create animals with significantly humanized brains or to bring about a human pregnancy in an animal uterus.

Part of the difficulty in coming to terms with these questions are the many variables involved. Research and therapeutic benefits that are often speculative must be weighed against risks, which depend in part on the nature of the animals being used, the times at which pluripotent cells are inserted into the developing organism, the likelihood of these cells integrating themselves into various animal tissues and organ niches, and whether the organism will be allowed to come to term. Compounding this complexity is the "irreducible degree of uncertainty about the cognitive nature of the new chimeric animal, and how it would manifest distress, anxiety, or other factors relevant to one's assessment of animal welfare" [74].

These complexities counsel case-by-case assessment of research directions, protocols based upon careful monitoring of outcomes, and the best evolving information about risks and benefits [66,79–81]. The International Society for Stem Cell Research (ISSCR) summarizes this position when it calls for "specialized oversight" of such research utilizing "baseline animal data grounded in rigorous scientific knowledge or reasonable inferences" and involving "a diligent application of animal welfare principles" [82]. In August 2015, the NIH imposed a moratorium on its funding of human-to-animal chimera research pending public comment on revised guidelines. These guidelines include the creation of a high-level internal NIH steering committee to provide broad policy oversight for this research. In addition, the guidelines include specific prohibitions on (1) inserting hESCs or hiPSCs into nonhuman primate blastocyst-stage embryos, and (2) breeding of animals where the introduction of hESCs or hiPCs may contribute to a germ line (i.e., make human eggs or sperm) [76]. A 2011 report by the British Academy of Medicine also recommends a prohibition on "substantial functional modification of the NHP [normal pressure hydrocephalus] brain, such as to engender 'human-like' behavior," though it offers somewhat more lenient permission, with oversight, for "substantial modification of an animal's brain that may make the brain function potentially more 'human-like,' particularly in large animals" [83]. Despite disagreements by national bioethics bodies at the margins of research possibilities, there is a consensus that chimera research involving the humanization of animal brains and any research that risks a human birth requires ongoing, case-by-case, specialist scrutiny of the scientific and ethical issues involved.

MAY WE GENETICALLY MODIFY HUMAN EMBRYOS?

Gene editing has recently been given new precision and applicability by development of CRISPR-Cas9 technology. It is now possible to target specific gene sequences in somatic cells, stem cells, gametes, or embryos for deletion or modification. This raises the prospect of human genetic engineering for the purpose of disease prevention and treatment and for genetic enhancement. For example, it might be possible to use genetically modified cells or stem cells to provide therapies for HIV AIDS or sickle cell anemia. Pathologies that begin at early uterine development might be prevented by the modification of parental gametes or the early embryo itself. Germline interventions like these have the added advantage of preventing future transmission of the disease-causing mutations. In many cases it is true, the same goal can be accomplished without gene editing by use of PGD and embryo selection, but gene editing permits the introduction of novel gene sequences not available in the parental lineage. It can also facilitate genetic enhancement, the creation of human beings who are "better than well" [27,28].

Despite its capabilities, CRISPR-Cas9 has deficiencies. It can alter and disrupt DNA at locations other than the intended ones ("off-target results"); it can change the DNA in some but not all, resulting in a mosaic of altered and unaltered cells, and can generate immune responses if introduced into the body. Although the CRISPR-Cas9 system is still undergoing development to reach the level of safety where it could be used in clinical applications, there is broad consensus that it would be premature and ethically questionable to attempt germline gene editing at this time, even for serious genetic diseases. In December 2015, leading scientists and bioethicists convened an international summit on human gene editing that concluded:

> It would be irresponsible to proceed with any clinical use of germline editing unless and until (i) the relevant safety and efficacy issues have been resolved, based on appropriate understanding and balancing of risks, potential benefits, and alternatives, and (ii) there is broad societal consensus about the appropriateness of the proposed application.

The report continues:

> At present, these criteria have not been met for any proposed clinical use: the safety issues have not yet been adequately explored; the cases of most compelling benefit are limited; and many nations have legislative or regulatory bans on germline modification. However, as scientific knowledge advances and societal views evolve, the clinical use of germline editing should be revisited on a regular basis.[84].

ARE THERE SPECIAL CONSIDERATIONS GOVERNING THE USE OF STEM CELLS IN CLINICAL RESEARCH AND CLINICAL APPLICATIONS?

Like all research or clinical translation involving the use of animal or human subjects, stem cell research is governed by a set of fundamental ethical principles that have been embodied in national and international codes and laws. As enunciated by the ISSCR, these principles include (1) research integrity; (2) primacy of patient welfare; (3) respect for research subjects; (4) transparency; and (5) social justice [82].

Some commentators have warned against "stem cell exceptionalism"—the singling out of stem cell research for unwarranted special attention [74]. Thus it may be asked whether the use of stem cells in research or clinical treatments raises any novel ethical questions. Given the controversial and moral status of hESCs and the long-lasting but still uncharted powers of stem cells themselves, it seems wise to add special provisions to the guidelines governing research and clinical applications when hESCs and hiPSCs are involved. Such guidelines have been developed by the Chief Medical Officer's Expert Group in Great Britain [85,86], by private ethics boards at the Geron Corporation [87] and Advanced Cell Technology [88] in the United States, by committees of the National Research Council and Institute of Medicine [66,80,81], by the NIH [50,89], and by the ISSCR [82,90,91]. These various guidelines tend to share several features.

Stem Cell Research Oversight/Embryo Research Oversight Committee Review

There is agreement that, in addition to the usual review of research proposals by an Institutional Review Board (IRB), it is appropriate that there be another layer of review provided by a stem cell research oversight (SCRO) committee, or, as it has more recently been termed, an embryo research oversight (EMRO) committee. The name change reflects the advent of hiPCs, the use of which, except for those having "organismal potential," raises fewer issues than the use of hESCs. In its most recent guidelines, the ISSCR recommends EMRO review for research involving human embryos, embryo-derived cells or that which entails "the production of human gametes in vitro when such gametes are tested by fertilization or used for the creation of embryos," and research on human totipotent cells that have the potential to sustain embryonic or fetal development [82]. In contrast, use of iPSCs requires human subjects review but does not require specialized EMRO review so long as the research does not generate human embryos or totipotent cells [82]. The ISSCR also exempts from EMRO review research with established hESC lines that are confined to cell culture or involve routine and standard research practice, such as assays of in vitro differentiation or teratoma formation in immune-deficient mice.

EMROs can operate at institutional or higher levels, and should include as members scientists and/or physicians not directly engaged in the research under consideration but with relevant expertise, ethicists, "members or advisors familiar with relevant local legal statutes governing the research," and "community members, unaffiliated with the institution through employment or other remunerative relationships, who are impartial and reasonably familiar with the views and needs of research subjects, patients and patient communities who could be benefited by stem cell research, and community standards" [82].

Donor and Procurement Issues

Because hESC and therapeutic cloning research require a supply of human gametes and embryos, any of which raise sensitive issues of moral status and familial relationships, steps must be taken to elicit the informed consent of donors, to protect their privacy, and to minimize any risks to which they might be subject. Because hiPCs are derived from human somatic material, existing IRB regulations concerning review and informed consent cover their derivation, and no EMRO committee review is needed. Nevertheless, the possibility of the use of such cells in chimeric animals and special transplantation issues recommend EMRO review in some cases. To aid researchers, the ISSCR has offered the outlines of a sample consent document for these purposes [82].

In general, explicit and contemporaneous informed consent (consent given at the time of procurement) is required when donors are asked to provide biomaterials for the creation of embryos, hESCs, or immortalized hiPC lines. Contemporaneous consent is not necessary if researchers procure somatic cells from a tissue bank, but in such cases the tissue bank's consent documents must evidence that donors consented to the use of their biomaterials for gamete creation or for stem cell research. There is wide consensus that donors of cells, sperm, or embryos should not be compensated, although it is regarded as permissible to provide compensation for the time, inconvenience, and discomfort associated with the donation of oocytes for research so long as payment does not vary according to the planned use of the oocytes, the number or quality of oocytes retrieved, the number or outcome of prior donation

cycles, or the donor's ethnic or other personal characteristics [70]. Payment levels should not be so high as to constitute an "undue influence" on a woman's decision-making.

Informed consent requires that donors fully understand the nature of the research being undertaken (for example, that it may require the destruction of an embryo), and that they explicitly consent to it. Whenever possible, donors should be given the option of agreeing to some forms of hESC research but not others (e.g., the creation of stem cells but not cloned embryos). It is morally impermissible to elicit somatic cells, sperm, eggs, or embryos for the production of stem cell lines without informing donors that an immortalized, pluripotent cell line might result that could be widely used in research or therapeutic applications. If there are likely to be commercial benefits flowing from the research, donors must also be informed of this, and their rights (if any) in such benefits should be clearly specified. If the research involves therapeutic cloning, both egg and somatic cell donors must be informed that a cloned embryo and a cell line with the egg donor's mitochondrial genetic material and the somatic cell donor's nuclear DNA will result. iPSC somatic cell donors should also be informed about the developmental and commercial possibilities of their cellular materials. Special questions also arise about the use of somatic cells, embryos, or sperm whose provenance is uncertain, including embryos created with sperm whose donor is unknown [92].

An emergent problem given the possible widespread use of hESCs and iPSCs is the discovery of incidental genetic findings in the course of downstream research that might be of health importance to donors. ISSCR and other bodies recommend the development of a policy on whether and how incidental findings will be returned to donors and the communication of this policy to donors at the time of the informed consent process. Successful implementation of this policy depends on the traceability of cell lines and strict compliance with material transfer agreements [82].

In conducting research, efforts should be made to preserve donor privacy by removing identifying information from gametes, embryos, and cell lines, and keeping this information apart in a secure location. In view of the controversy surrounding much of this research, donors can be subjected to harassment or embarrassment if their association with the research is revealed without their consent. In an era where it is increasingly possible to identify donors from the DNA of the resulting stem cells, donors should be warned of possible risks to their privacy and that of their family members.

Finally, there is a question about the use of hESC lines previously produced (or produced abroad) under less comprehensive guidelines. The NIH faced this question in its effort to develop guidelines following President Obama's executive order permitting funding for hESC research. Many hESCs available for use had not been developed in precise conformity to the newly developed guidelines. The NIH met this problem by forming a Working Group of the Advisory Committee to the Director to consider exceptions that conform to the spirit of the guidelines. Rather than "grandfathering" preexisting hESCs, the Working Group is empowered to judge hESC lines "derived in a responsible manner" to be eligible for use in NIH-funded research [50].

Research Conduct

Guidelines also apply to the overall conduct of research. These include the requirement that no embryo used in hESC or therapeutic cloning research be allowed to develop beyond 14 days. This internationally recognized limit is based on the substantial changes that occur at gastrulation, which marks the beginning of individualization and organogenesis [93]. At this time, ISSCR guidelines also prohibit reproductive cloning research; research in which human embryos (or organized cellular structures that might manifest human organismal potential) are gestated ex utero or in any nonhuman animal uterus, or have undergone modification of their nuclear genome and are implanted into or gestated in a human or animal uterus; and research in which animal chimeras incorporating human cells with the potential to form human gametes are bred to each other [82]. Supervision and accountability of all staff and scientists involved in this research to prevent any diversion of gametes or embryos to reproductive purposes is required.

Clinical Translation

Stem cell-based treatments have been the clinical standard of care for some conditions, such as hematopoietic stem cell transplants for leukemia, but research on stem cell-based therapies has greatly expanded in recent years. Investigational New Drug applications have been filed for several therapies, and clinical trials are under way. As might be expected, problems have already developed. In late 2009, the Food and Drug Administration (FDA) asked the Geron Corporation to halt its trial on a groundbreaking stem cell therapy for spinal cord injury when a review revealed the formation of cysts in some (animal) trial subjects [94].

All tissue transplantation involves risks for the recipient, but for several reasons these risks are magnified in the case of stem cell transplants:

- Stem cells are novel therapeutic agents whose development and manufacture require innovative procedures for ensuring purity and homogeneity. Additional risks derive from the fact that cells are likely to be expanded in culture and/or exposed to xenoculture materials, viruses, or other infectious agents before transplantation. The likely use of these cells by large populations increases the need for careful oversight in cells' development and manufacture.
- Cell types may differ in their ability to proliferate and safely implant in the body. Their use poses risks of infusional toxicity, severe immune responses, and tumorigenesis. An additional problem is ectopic or off-target insertion.
- Animal models may not accurately reflect toxicity in humans.

In response to these challenges, in 2008 a multidisciplinary, international task force of the ISSCR proposed ethical guidelines for clinical translational research. The task force issued 39 recommendations, most of which fall into five broad categories: (1) cell processing and manufacture, (2) preclinical studies, (3) clinical research, (4) stem cell-based medical innovation, and (5) considerations of social justice [91].

With regard to cell processing and manufacture, the task force recommended that these be conducted under "scrupulous, expert, and independent review and oversight." As a rule, minimally manipulated products (cells maintained in culture under nonproliferating conditions for short periods of time) require less oversight than those subjected to extensive manipulations, such as genetic alterations. The same is true of autologous versus allogenic use and use for homologous versus nonhomologous functions (Recommendations 8 and 9). Good manufacturing practice must be applied to manipulated products and those destined for allogeneic use. Donors of cells should be screened for infectious diseases and the donor should give written informed consent that covers the likely storage, future manipulations, analyses, uses of their cells, commercial potential, and possible risks to the donor's privacy, including exposure of genetic information (Recommendation 3).

Preclinical studies are meant to provide evidence of product safety and proof-of-principle of therapeutic effect. This normally requires sufficient studies in animal models, including larger animals where structural tissue needs to be tested in a load-bearing model (Recommendations 11 and 14). Researchers must develop and implement a clear plan to assess the risks of tumorigenicity for any cell product (Recommendation 18). Cell cultures and animal models should be used to test the interaction of cells with drugs to which recipients will be exposed, including the immunosuppressants planned for recipients (Recommendation 19).

Clinical trials of stem cell research must conform to internationally accepted principles governing the protection of human subjects, including regulatory oversight, peer review by an expert panel independent of the investigators and sponsors, fair subject selection, informed consent, and patient monitoring. In addition, there are special issues raised by stem cell research. Because of their undetermined risks stem cell-based therapies, as a rule, must aim at being clinically competitive or superior to existing therapies. Where there are already efficacious therapies for a disease condition, the stem cell-based intervention must be of low risk and offer a potential advantage (such as better functional outcome; single procedure vs. lifelong drug therapy). Greater risks are permissible where there is no efficacious therapy and where the disease condition is severely disabling and life threatening (Recommendation 25). Patients need to be informed that cell-derived products have never been tested before in humans, that researchers do not know whether they will work as hoped, and that, unlike many pharmacological products or even many implantable devices, stem cells may stay in the body and generate adverse effects for the lifetime of the patient. To respect their values, and because some subjects may have moral objections to the use of embryo-derived cells, subjects should be informed about the source of the cells (Recommendation 28). To determine the consequences of cellular implantation, and with consideration of cultural and familial sensitivities, subjects should be asked for autopsy in the event of death (Recommendation 31).

Considerations of social justice apply to all research involving human subjects but receive special importance in view of public involvement in this emergent research area. Among the recommendations of the task force are public engagement in the policy making of governmental agencies (Recommendation 37), fair allocation of benefits and risks, and the need to develop alternative models of intellectual property, licensing, product development, and public funding to promote fair and broad access to the new diagnostics and therapies (Recommendation 38). One justice consideration especially pertinent to stem cell research is the establishment of stem cell collections with genetically diverse sources of cell lines (Recommendation 38). The task force concludes its recommendations with an aspirational goal that companies, subject to their financial capability, should offer affordable therapeutic interventions to persons living in resource-poor countries who would not otherwise have access to these therapies.

Universities and other institutions are asked to incorporate this requirement in their intellectual property licenses (Recommendation 39).

Clinical Application

Recommendations for the ethical introduction of stem cell therapies take place against a background marked by the presence of many clinical programs offering "stem cell therapies" outside the boundaries of proper clinical trials and ethical or legal oversight [95]. These unsupervised stem programs exploit a loophole in the FDA regulation of biologics that permits the use of cells that have been subject to "minimal manipulation" [96]. In some cases desperate patients have suffered serious illnesses, including growths caused by proliferating and ectopic transplanted stem cells [97]. In its 2016 report, the ISSCR issues a stern rebuke to such programs.

> The ISSCR condemns the administration of unproven stem cell-based interventions outside of the context of clinical research or medical innovation compliant with the guidelines in this document and relevant laws, particularly when it is performed as a business activity. Scientists and clinicians should not participate in such activities as a matter of professional ethics. For the vast majority of medical conditions for which putative "stem cell therapies" are currently being marketed, there is insufficient evidence of safety and efficacy to justify routine or commercial use [82].

The ISSCR guidelines do not altogether prohibit innovative clinical therapies. They affirm that "unproven stem cell-based interventions" can be provided to at most a very small number of patients outside the context of a formal clinical trial but in accordance with a set of "very restrictive conditions." These include the existence of a written plan outlining the scientific rationale and justification of the procedure, why it has a reasonable chance of success, and why it should be attempted compared to existing treatments. This plan should offer a full characterization of the types of cells being transplanted, how they will be administered, and a commitment to clinical follow-up and data collection to assess the procedure's effectiveness and adverse effects. The plan should be peer reviewed by independent experts, and have the full support and accountability of the health care institution where it is based. Patients must provide voluntary informed consent and understand that the intervention is unproven. There should be an action plan for adverse events, including insurance coverage for patients. Finally, the conduct of innovative treatments requires a commitment by clinician-scientists to use their experience with individual patients to contribute to generalizable knowledge. This includes communication of all outcomes to the scientific community in professional meetings and journals, and timely movement to formal clinical trials [82].

CONCLUSION

Fully answering all of the questions this chapter has raised would require an ethical treatise. However, in my view, the moral claims of the very early embryo do not outweigh those of children and adults that can be helped by hESC and therapeutic cloning technologies. While iPSC research is promising, reliance on it should not replace the use of hESC lines produced through proven techniques, and that are currently available. As we move forward to clinical translational research, care should be taken that there is proper oversight for these novel and potentially harmful therapies. Some may disagree with these conclusions. Continuing dialog about these questions and clearer scientific research results will bring us closer to a consensus on these issues.

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CHAPTER

77

Overview of the US Food and Drug Administration Regulatory Process

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INTRODUCTION AND CHAPTER OVERVIEW

The field of regenerative medicine encompasses a breathtaking array of interdisciplinary scientific approaches that address a broad spectrum of clinical needs. Ongoing advances in scientific knowledge related to cell biology, gene transfer therapy, biomaterials, immunology, engineering principles, and technology applicable to biological systems foster innovation of regenerative medicine products. This places the regenerative medicine community in a position to address a number of challenging and critical health needs including the treatment of disease conditions resulting from pancreas, liver, and kidney failure; structural cardiac valve repair; skin and wound repair; and orthopedic applications, among others. Scientific challenges confronting this field include expanding the knowledge base in each discipline as well as developing an interdisciplinary approach for identifying and resolving key questions. The US Food and Drug Administration's (FDA's) regulatory review process mirrors the scientific challenges with regard to the development of review paradigms that cross scientific disciplines.

This chapter will provide a brief historical review of the FDA and its organizational structure and discuss topics pertaining to the regulation of regenerative medicine products including possible regulatory pathways for combination products and relevant jurisdictional issues. Sources of information concerning FDA regulatory policies important to regenerative medicine product developers will also be discussed. It is essential for individuals, institutions, and companies responsible for the clinical trials of regenerative medicine products to be aware of FDA regulatory policies and how to obtain the necessary information. These entities are collectively referred to in FDA regulations as Sponsors (the term "Sponsor" for drugs and biologics is defined in 21 Code of Federal Regulations [CFR] 312.3[b] whereas "Sponsor" is similarly defined in 812.3[n] for devices). Suggestions will also be provided regarding how to engage FDA effectively during the development of a novel regenerative medicine product.

BRIEF LEGISLATIVE HISTORY OF UNITED STATES FOOD AND DRUG ADMINISTRATION

Medical products regulated by the FDA include human and animal drugs, medical devices, and biological products such as vaccines, cellular and gene therapies, and blood products. Among the therapeutic agents of biological origin regulated by the FDA are cellular therapies, including products derived in whole or part from human tissue, and xenotransplants. In addition to medical products for human use, the FDA regulates food other than most meat and poultry; radiation-emitting products for consumer, medical, and occupational use; cosmetics; medical products

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for veterinary use; and animal feed. The FDA's role in medical product regulation extends throughout the entirety of the product life cycle. Depending on the product category, this may mean oversight, including review and inspection, of clinical trials, of the premarket product approval/clearance process, of manufacturing controls, controls over labeling, and registration and listing requirements. After a product is marketed, the FDA also continues its oversight in a variety of ways, including inspections, mandatory and voluntary postapproval (e.g., Phase IV) studies, and surveillance of adverse events reported to the FDA.

FDA laws and regulations have developed over time, partly in response to serious medical adverse events or by other public health and safety concerns. Early regulation of biological products was prompted in part by the death of 13 children in 1901 after the administration of diphtheria antitoxin prepared from a source contaminated with tetanus. In response, Congress passed the Biologics Control Act in 1902. This act provided for regulation of viruses, serums, toxins, and analogous products; required licensing of manufacturing establishments and manufacturers; and provided the government with inspectional authority. The Act focused on requiring control of manufacturing processes for producing biological products, reflecting the extent to which the starting source material and the manufacturing process defined the final product.

In 1906, Congress passed the Federal Food and Drugs Act, proposed in part in reaction to the meat-packing industry conditions described in Upton Sinclair's book, *The Jungle*. Although the primary focus of the Act was on food safety, the law also required that drugs be provided in accordance with standards of strength, quality, and purity unless otherwise specified in the label. Premarket review of new drugs was not required until the passage of the 1938 Food, Drug, and Cosmetic Act (FD&C Act), which repealed the earlier 1906 Federal Food and Drugs Act. In 1937, the sulfa drug Elixir Sulfanilamide, previously available only in tablet or powder form to treat streptococcal infections, was marketed as a liquid using diethylene glycol, an analog of antifreeze, as a formulating solvent. This change in formulation, which was made without the requirement for premarket review, resulted in over 100 deaths, many of whom were children, and prompted the passage of the 1938 FD&C Act. The 1938 Act also put medical devices and cosmetics under FDA authority and authorized factory inspections.

The Public Health Service Act (PHS Act), passed in 1944, incorporated the 1902 Biologics Control Act and is the current legal basis for the licensing of biological products. Because most biological products also meet the definition of "drugs" under the FD&C Act, they are also subject to regulation under that Act.

The requirement for premarket demonstration of efficacy and the authority for FDA oversight of clinical trials were provided by the Kefauver–Harris amendments to the FD&C Act in 1962. These amendments were prompted in part by the tragic adverse events resulting from use of thalidomide as a nonaddictive prescription sedative. This drug, which was not approved as a sedative in the United States, resulted in thousands of birth defects in children born outside this country when taken by pregnant women during the first trimester.

The Medical Device Amendments to the FD&C Act were passed in 1976, after reports of safety issues with respect to the Dalkon Shield intrauterine device. The Medical Devices Amendments included risk-based requirements for premarket notification or approval of medical devices. Before 1976, FDA authority was limited to taking action against marketed devices found to be unsafe or ineffective.

LAWS, REGULATIONS, AND GUIDANCE

The previous section summarized the history of laws that form the underpinning of FDA medical product regulation. This section provides a brief description of how laws are made and implemented, the procedures for promulgating regulations, and a description of how the FDA develops and uses guidance documents.

Laws are created as an outcome of legislative activity conducted in the US Senate and House of Representatives resulting in passage of a bill. Once Congress passes a bill, it becomes law if it is signed by the president. If the president vetoes the bill, it becomes law if two-thirds of the Senate and House of Representatives vote in favor of the bill. A federal law also is denoted as a public law and may contain a name, such as the FD&C or PHS Acts. These laws are then incorporated into the US Code (USC), which is updated every 6 years, with supplements published regularly to incorporate changes to statutes between updates. Drug, biologic, and device laws can be found in the USC at:

- Drugs and Devices: Title 21 Chapter 9, and
- Biologics: Title 42 Chapter 6A.

When laws are passed, government agencies such as the FDA often implement them by promulgating regulations. Sometimes an agency may elect to promulgate regulations on its own, whereas other laws may explicitly require an agency to issue regulation. The process for making regulations must be performed in accordance to the Administrative Procedures Act (Title 5, USC, Chapter 5). This Act generally requires agencies such as the FDA to provide public notice and opportunity for comment as part of the rule-making process.

FDA regulations are contained in the CFR. Regulations for drugs, biologics, devices, tissues, and combination products, along with related regulations, may be found in various parts of Title 21 of the CFR. The following is a list of key regulatory provisions:

- Drugs: 21 CFR Parts 200–299, 300–369;
- Biologics: 21 CFR Parts 600–680;
- Devices: 21 CFR Parts 800–898;
- Human Cells, Tissues, and Cellular and Tissue-based Products (HCT/P): 21 CFR Parts 1270/1271;
- Combination Products: 21 CFR Parts 3 and 4;
- Recalls: 21 CFR Part 7;
- Informed Consent/Institutional Review Boards (IRBs): 21 CFR Parts 50/56;
- Financial Disclosure by Clinical Investigators: 21 CFR Part 54;
- Good Laboratory Practice for Nonclinical Laboratory Studies: 21 CFR Part 58; and
- Good Guidance Practices: 21 CFR Part 10.115;

Guidance documents are nonbinding publications that describe the FDA's interpretation of policy pertaining to a regulatory issue or set of issues related to:

- the design, production, labeling, promotion, manufacturing, and testing of regulated products;
- the processing, content, and evaluation or approval of submissions; and
- inspection and enforcement policies.

Guidance documents, which are developed in accordance with Good Guidance Practices found at 21 CFR 10.115, are intended to clarify the FDA's current thinking related to regulatory issues and procedures. Unlike regulations and laws, guidance documents are not enforceable. Therefore, Sponsors may elect to choose alternate approaches that still comply with existing laws and regulatory requirements. In most cases, guidance documents are issued in draft form for public comment before implementation. In cases in which prior public participation is not feasible or appropriate, the FDA may issue a guidance document for immediate implementation without first seeking public comment. The publication of guidance documents reflects the FDA's efforts to convey up-to-date information on general and cross-cutting topics to those involved in the developing field of regenerative medicine.

When considering developing a guidance document, the FDA may freely discuss related issues with the public. In fact, the FDA may hold a public meeting, advisory committee meeting, or workshop to obtain input on scientific issues. Finally, after receiving public input, the FDA will evaluate submitted comments and finalize the document. Guidance documents are a useful way for the FDA to communicate current thinking to the public. Within the arena of regenerative medicine, it is valuable to be aware of both product-specific and cross-cutting guidance documents. Some of the more pertinent guidance documents to this field, such as those related to preclinical testing, manufacturing practices, and clinical trial design, are discussed in this chapter. In addition to guidance documents, the FDA may refer to guidelines published by the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH). ICH is an international effort to harmonize regulatory requirements. ICH guidelines, similar to FDA guidance documents, are nonbinding.

FOOD AND DRUG ADMINISTRATION ORGANIZATION AND JURISDICTIONAL ISSUES

Scientific development of regenerative medicine products involves extensive testing and planning before marketing authorization. It can be helpful for individuals and organizations involved in product development to engage in early dialog with the appropriate FDA review unit to receive and consider the FDA's comments on the design of the preclinical and clinical development plan. This section describes the FDA's organizational structure and provides basic information regarding jurisdictional decisions made to determine the appropriate regulatory pathway for a broad range of products.

The FDA is an agency within the Department of Health and Human Services (DHHS) consisting of seven centers and the Office of the Commissioner. Three of the centers are responsible for regulating medical products for humans. The Center for Biologics Evaluation and Research (CBER) has jurisdiction over a variety of biological products, including blood and blood products, vaccines and allergenic products, and cellular, tissue, and gene therapies, as well as some related devices. The Center for Devices and Radiological Health (CDRH) has jurisdiction over diagnostic and therapeutic medical devices, administration of the Mammography Quality Standards Act program, and ensuring the safety of radiation-emitting products. The Center for Drug Evaluation and Research (CDER) has jurisdiction over a variety of drug products, including small molecule drugs and well-characterized biotechnology-derived therapeutic biological products that include monoclonal antibodies and cytokines.

For many medical-use products, it is clear which center within the FDA should have primary jurisdiction for the premarket review. For other products, including some technologically novel products under development, determining which center has jurisdiction for review may be unclear. Important starting points for determining product jurisdiction are the formal regulatory definitions of biological products, drugs, devices, and combination products. The formal definitions are as follows:

- Biological Product (42 USC 262[i](1)): A virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, protein (except any chemically synthesized polypeptide) or analogous product, or arsphenamine or derivative of arsphenamine (or any other trivalent organic arsenic compound), applicable to the prevention, treatment, or cure of a disease or condition of human beings.
- Drug (21 USC 321[g] [1]): (A) Articles recognized in the official US Pharmacopeia, official Homeopathic Pharmacopeia of the United States, or official National Formulary, or any supplement to any of them; and (B) articles intended for use in the diagnosis, cure, mitigation, treatment, or prevention of disease in man or other animals; and (C) articles (other than food) intended to affect the structure or any function of the body of man or other animals; and (D) articles intended for use as a component of any articles specified in clause (A), (B), or (C).
- Device (21 USC 321[h]): An instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent, or other similar or related article, including any component, part, or accessory, which is: (1) recognized in the official National Formulary, or the US Pharmacopeia, or any supplement to them, (2) intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment, or prevention of disease, in man or other animals, or (3) intended to affect the structure or any function of the body of man or other animals, and which does not achieve its primary intended purposes through chemical action within or on the body of man or other animals and which is not depend on being metabolized for the achievement of its primary intended purposes.
- Combination Product (21 CFR 3.2[e]): (1) A product composed of two or more regulated components, that is, drug–device, biologic–device, drug–biologic, or drug–device–biologic, that are physically, chemically, or otherwise combined or mixed and produced as a single entity; (2) two or more separate products packaged together in a single package or as a unit and composed of drug and device products, device and biological products, or biological and drug products; (3) a drug, device, or biological product packaged separately that according to its investigational plan or proposed labeling is intended for use only with an approved individually specified drug, device, or biological product where both are required to achieve the intended use, indication, or effect and where upon approval of the proposed product the labeling of the approved product would need to be changed, for example, to reflect a change in intended use, dosage form, strength, route of administration, or significant change in dose; or (4) any investigational drug, device, or biological product packaged separately that according to its proposed labeling is for use only with another individually specified investigational drug, device, or biological product where both are required to achieve the intended use, indication, or effect.

The FDA's Office of Combination Products (OCPs), located in the Office of the Commissioner, has broad administrative overview responsibilities covering the regulatory life cycle of drug–device, drug–biologic, device–biologic, and drug–biologic–device combination products. When jurisdiction is uncertain, Sponsors may contact the OCP for assignment of primary regulatory review responsibility for combination and other medical products. Jurisdictional determinations are made after a formal submission process called a Request for Designation. The appropriate FDA center jurisdiction is determined by considering the primary mode of action of the product.

APPROVAL MECHANISMS AND CLINICAL STUDIES

There are several premarket approval pathways for medical products, depending on whether the product is a drug, biological product, or device. Approval pathways, explained in more detail subsequently, include the New Drug Application (NDA) for drugs and the Biologics License Application (BLA) for biologics. The Premarket Approval Application (PMA), Humanitarian Device Exemption (HDE), 510(k) clearance mechanism, and De Novo classification process are various regulatory pathways used for medical devices. Clarification regarding the type of application needed for a particular regenerative medicine product may be helpful to the Sponsor early in development to enable the Sponsor to discuss the data needed for a marketing application during the planning stage.

NDAs and BLAs are applications for licensure under the safety and effectiveness requirements of the FD&C Act. Biologics are further subject to the approval standards set forth in the PHS Act that requires demonstration that the product is safe, pure, and potent. Further information concerning the licensure of drugs and biological products is

provided in "Guidance for Industry: Providing Clinical Evidence of Effectiveness for Human Drugs and Biological Products" [1]. For a medical device, the appropriate regulatory pathway and classification depend heavily on the associated risks; Class I includes devices with the lowest risk and Class III includes devices with the highest risk. A PMA is an application for approval for most Class III medical devices; the Sponsor must show reasonable assurance of safety and effectiveness [2]. The premarket notification, or 510(k), clearance process applies to products that are "substantially equivalent" to a Class I or II (or in a few cases, a Class III) medical device already on the market [3]. The De Novo process provides a pathway to Class I or Class II classification for medical devices for which there is no legally marketed device to enable a demonstration of "substantial equivalence" but for which regulatory controls provide a reasonable assurance of safety and effectiveness [4]. Under medical device regulation, a product can also gain approval through an HDE, which is a marketing approval for certain devices for rare conditions and is exempt from the effectiveness requirements of a PMA but requires demonstration of safety and probable benefit [5]. To qualify for this type of application, a Sponsor would first need to receive a designation from the FDA Office of Orphan Products Development that the medical device is a Humanitarian Use Device intended for treatment or diagnosis of a disease or condition that affects or is manifested in not more than 8000 individuals per year in the United States.

Many but not all combination products are approved or cleared under a single marketing application. For example, depending on the specific facts, including the primary mode of action of the product, a combination biological device could be licensed under the biologics authorities or approved under the medical device authorities. After approval of a marketing application, there are also postmarketing requirements such as reporting [6].[1,2] In addition, modifications to the product or labeling may require prior approval. The FDA has published regulations and guidance documents that address submission and approval processes for modifications to marketed products [3,6].[3,4,5] Compliance with manufacturing requirements is also an ongoing Sponsor obligation. The FDA has issued a guidance document entitled "Guidance for Industry and FDA Staff: Current Good Manufacturing Practice Requirements for Combination Products," which provides direction regarding applicable manufacturing requirements for combination products [7]. Owing to the relatively new nature of regenerative medicine and its developmental status, postapproval topics will not be further discussed in this chapter.

When clinical investigation is needed to evaluate the safety and efficacy of an investigational product before marketing approval, an Investigational New Drug (IND) application is required for drugs and biologics and an Investigational Device Exemption (IDE) is generally required for devices [8,9]. For both types of applications, the Sponsor needs to submit a description of the product and manufacturing process, preclinical studies, a clinical protocol, information on any other prior investigations such as human clinical studies, and a rationale for the study design. An IRB review and informed consent are also required. The FDA has 30 days to review the application to determine whether the study may proceed. The contents are specifically laid out in FDA regulations for each type of application. Requirements for the content of an IND can be found at 21 CFR 312.23, and for an IDE at 21 CFR 812.20.

For some products, there may be applicable guidance with respect to the manufacturing information and the preclinical data needed to support the study. For example, "Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs)" provides information on characterization and manufacturing of a cellular product to be submitted in an IND [10]. Information on a general preclinical study design for regenerative medicine products using cellular or gene therapies can be found in the "Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products" [11]. For medical devices, "Guidance for Industry and FDA Staff, Investigational Device Exemptions (IDEs) for Early Feasibility Medical Device Clinical Studies, Including Certain First in Human (FIH) Studies" [12] provides information on early feasibility study IDEs that allow for early clinical evaluation of significant risk devices. Early feasibility study IDEs may be appropriate early in device development when clinical experience is necessary because nonclinical testing methods are not available or adequate to provide the information needed to advance the developmental process. Applicable regulations and guidance should

[1] Reporting for Biological Products: 21 CFR 314.80 and 21 CFR 314.81, 21 CFR 600.14, 21 CFR 600.80, 21 CFR 600.81, 21 CFR 601.28, 21 CFR 601.70, and 21 CFR 601.93.

[2] Postmarketing Reports for Applications for FDA Approval to Market a New Drug (NDA): 21 CFR 314.80 and 314.81.

[3] PMA supplements: 21 CFR 814.39. (2007).

[4] Supplements and Changes to an Approved NDA: 21 CFR 314.70, 314.71, 314.72. (2007).

[5] BLA 21 CFR 814.39, 21 CFR 314.70, 21 CFR 314.71, 21 CFR 314.72.

be further consulted for information on adverse event reporting, labeling, study conduct and monitoring, and other topics related to requirements for conducting an IND [8] and IDE [13–15]. For information on general clinical study design and conduct issues, the FDA has many guidance documents that may be helpful [16,17]. For some indications there may be guidance documents that apply across technologies, such as the "Guidance for Industry: Chronic Cutaneous Ulcer and Burn Wounds—Developing Products for Treatment" [18] and "Guidance for Industry: Cellular Therapy for Cardiac Disease" [19]. In addition, guidance documents not directly applicable for a specific product, indication, or technology may be worth consulting, because the documents may provide some insights into general clinical issues, such as assessment parameters that may be valuable.

MEETINGS WITH INDUSTRY, PROFESSIONAL GROUPS, AND SPONSORS

Although the terminology and procedures may vary, all three FDA centers performing medical product review encourage meetings with Sponsors to address questions before a regulatory submission and at specific developmental milestones. When requesting a formal or informal meeting with the FDA, it is helpful to provide background information as well as specific discussion questions. Further information about formal meetings, such as what to include in a meeting request and what type of information to include in the briefing package submitted before the meeting, is provided in "Guidance for Industry: Formal Meetings Between the FDA and Sponsors or Applicants" [20] and "Guidance for Industry and FDA Staff, Requests for Feedback on Medical Device Submissions: The Pre-Submission Program and Meetings with Food and Drug Administration Staff" [21]. Early-stage device meetings are addressed in "Early Collaboration Meetings Under FDA Modernization Act (FDAMA): Final Guidance for Industry and CDRH Staff" [22].

The FDA also interacts with organizations representing a group of interested parties (e.g., the International Society for Cellular Therapy, the American Association for Blood Banks, the Biotechnology Industry Organization, and Pharmaceutical Research and Manufacturers of America), which provides an opportunity to discuss topics of interest to the FDA and the organization. These interactions can be valuable for the FDA and stakeholders because they are a way to understand general issues of concern better, as opposed to product-specific discussions with individual firms. In addition to such interactions and meetings with individual sponsors, the FDA has various advisory committees that review available data and information and make recommendations related to a variety of issues, many of which are pertinent to the field of regenerative medicine. Advisory committees will be discussed further in the Advisory Committee Meetings section.

REGULATIONS AND GUIDANCE OF SPECIAL INTEREST FOR REGENERATIVE MEDICINE

The topics discussed thus far have been of general applicability to medical product regulation: marketing pathways, clinical trial regulation, meetings, guidance development, and related topics. This section will review a few topics of particular interest to the scientific community engaged in developing regenerative medicine products: FDA regulations of human tissue products, product characterization for cellular products, FDA policy and guidance on xenotransplantation, and gene therapy.

Regulation of Human Cells and Tissues Intended for Transplantation

An understanding of the regulations applicable to cells and tissues is important for developers of regenerative medicine products because human cells or tissues comprise the whole of many products or are a key component of them.

In 1997, noting the fragmented approach to the regulation of human cellular and tissue-based products, the FDA issued the "Proposed Approach to the Regulation of Cellular and Tissue-Based Products" [23]. This document proposed a tiered risk-based approach to regulating these products. According to the proposed approach, products posing a lesser degree of risk would be subject to the rules designed to minimize communicable disease risks and additional regulatory requirements would be imposed on products posing an additional risk. The proposed approach to regulating human tissues was implemented in three parts, collectively referred to as the "tissue rules": Establishment Registration and Listing, Donor Eligibility, and Current Good Tissue Practice (CGTP). The complete

set of rules went into effect on May 25, 2005 and is codified in 21 CFR Part 1271. The tissue rules derive from the statutory authority of section 361 of the PHS Act, which addresses the control of spread of communicable diseases. Because the tissue rules apply to all human cellular and tissue-based products, it is important for Sponsors of regenerative medicine products to be aware of these rules, as well as the specific additional requirements for biologics or devices that may apply, depending on the particular regulatory pathway for their products.

With some exceptions that are noted in the tissue rules, human cells or tissue intended for implantation, transplantation, infusion, or transfer into a human recipient are regulated as an HCT/P. Examples of HCT/Ps are musculoskeletal tissue, skin, ocular tissue, heart valves, dura mater, reproductive tissue, gestational tissue such as umbilical cord tissue and amniotic membrane, and hematopoietic stem/progenitor cells. Specifically excluded are vascularized organs, minimally manipulated bone marrow, blood products, xenografts, secreted or extracted products such as human milk and collagen, ancillary products, and in vitro diagnostic products.

Tissue rules require HCT/P establishments to do the following:

- register and list their HCT/Ps with FDA (21 CFR 1271 Subparts A and B);
- evaluate donors through screening and testing to reduce risk for transmission of infectious diseases through transplantation (21 CFR 1271 Subpart C); and
- follow CGTP to prevent the spread of communicable disease (21 CFR 1271 Subpart D).

Additional requirements for reporting, labeling, inspections, importation, and enforcement are described in 21 CFR 1271 Subparts E and F; these provisions apply only to HCT/Ps regulated solely under Section 361 of the PHS Act, and therefore would not apply to most regenerative medicine products.

The Establishment Registration rule defines the circumstances under which a product would be subject to the HCT/P rules only (21 CFR 1271.10), and when there would be additional regulatory oversight such that a BLA, PMA, or other marketing application would be required (21 CFR 1271.20). Products that meet all of the following conditions are regulated by the FDA solely under the HCT/P rules: (1) the HCT/P is not more than minimally manipulated, (2) the HCT/P is intended for homologous use, (3) the HCT/P is not combined with a drug or device (with certain exceptions), and (4) the HCT/P does not have a systemic effect and is not dependent upon the metabolic activity of living cells for its primary function (except for autologous use or allogeneic use in a first or second degree blood relative, or reproductive use). If any of these four conditions are not met, a marketing application is required. The Tissue Reference Group handles various inquiries from stakeholders concerning application of the HCT/P rules including generating recommendations for consideration for CBER, CDRH, and OCP regarding whether specific HCT/Ps meet the criteria specified in 21 CFR 1271.10 for regulation solely under Section 361 of the PHS Act. Additional information and documents regarding these rules, as well as electronic forms for registration and listing, can be found on the FDA's website for Tissue and Tissue Products [24].

A joint FDA—Centers for Disease Control and Prevention (CDC) workshop held in 2007 on the Processing of Orthopedic, Cardiovascular, and Skin Allografts is relevant to the regenerative medicine field. The workshop discussed pertinent information regarding current clinical practices, experiences, expectations, and assumptions of safety when using tissue allografts; the challenges of processing allografts with a focus on disinfection and sterilization methods; and the usefulness, reliability, and validation of tissue culturing methods. Some manufacturing topics discussed at the workshop are addressed in "Guidance for Industry: Current Good Tissue Practice (CGTP) and Additional Requirements for Manufacturers of HCT/Ps" [25].

The FDA issued "Guidance for Industry: Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products (HCT/Ps)" to assist establishments in making donor eligibility determinations with compliance to the Donor Eligibility rule (21 CFR 1271 Subpart C) [26]. Since 2007, multiple guidance documents have been published to provide updated or new recommendations for HCT/P donor screening and donor testing as well as guidance involving regulatory considerations for HCT/Ps from adipose tissue, to meet minimal manipulation criterion, interpret and apply homologous use, and interpret the scope of the same surgical procedure exception under 21 CFR 1271.15(b) [27].

Human Cellular Therapies

Although therapeutic products composed of or containing cells vary greatly in the specific details of their application, cell or tissue source, manufacturing process, and characteristics, there are regulatory considerations that apply to all cellular preparations being developed as investigational regenerative medicine products intended for early-phase clinical studies. Control of the source material, demonstrated control of the manufacturing process,

and characterizations of the cellular product that results from the manufacturing process are three such safety and quality considerations and will be discussed briefly next.

The cell source will vary for different products and may be autologous or allogeneic, undifferentiated stem or progenitor cells, or terminally differentiated cells. Ensuring the safety of source cellular materials used during manufacture of an investigational regenerative medicine product begins by determining the eligibility of the donors selected to provide the source material through screening and testing. This screening and testing are part of the tissue rules described earlier in this chapter. Autologous products are not required to comply with the donor screening and testing requirements in the tissue rules but they carry labeling requirements to communicate risks for infectious substances. However, if autologous tissue either is positive for specific pathogens or has not been screened or tested, it is recommended that manufacturers document whether tissue culture methods could propagate or spread viruses or other adventitious agents to persons other than the recipient [10]. Donor eligibility determination is required for all allogeneic donors of cells and tissues.

There are other aspects of the cell source may raise substantial concerns. In addition to screening and testing donors for communicable disease agents, according to the "ICH Guidance on Quality of Biotechnological/Biological Products: Derivation and Characterization of Cell Substrates Used for Production of Biotechnological/Biological Products," the FDA has suggested that Sponsors consider the importance of evaluating donor medical history information and the relevance of conducting specified molecular genetic testing as part of an overall comprehensive assessment program to establish the suitability of a specific cellular preparation for use in manufacturing a regenerative medicine somatic cellular product [28]. The rationale for and feasibility of collecting additional information about molecular genetic testing were discussed in a public meeting of the FDA Biological Response Modifiers Advisory Committee (now known as the Cellular, Tissue, and Gene Therapies Advisory Committee [CTGTAC]) convened on Jul. 13–14, 2000, on the topic of "Human Stem Cells as Cellular Replacement Therapies for Neurological Disorders" [29]. A description of the physiological source of the cellular material, including the tissue of origin and phenotype such as hematopoietic, neuronal, fetal, or embryonic, conveys important information about the cells and their critical attributes.

Control of the manufacturing process provides assurance about the consistent, reproducible production of the cellular component. Often, manufacturing will involve a multistep process that must be performed using aseptic techniques to prevent introduction of microbial contamination [30]. In addition to these precautionary techniques, the final product resulting from the manufacturing process should be demonstrated to be free of viable contaminating organisms. For manufacturing processes that involve in vitro cell culture, the cell product should also be tested for mycoplasma contamination, which may be introduced by manufacturing reagents or the culture facility environment [31]. A final rule became effective on Jun. 4, 2012, which amended the sterility regulations in part to provide manufacturers of biological products with greater flexibility, as appropriate, and encourage use of scientific and technological advances in sterility test methods as they become available.[6]

Many types of reagents may be used to manufacture the cellular component of a product, including those that promote cellular replication or induce differentiation, and those used to select targeted cell populations, specifically serum, culture medium, peptides, cytokines, and monoclonal antibodies. It is possible that the manufacturing process uses reagents that are FDA approved or cleared, clinical grade, or research grade. Depending on the grade of a particular reagent, additional documentation may be needed to verify the source, safety, and performance of the reagent. For example, some materials, such as serum, may be human- or animal-derived products, which have an increased risk for containing adventitious agents and therefore require further documentation of the safety testing performed on each lot of material. It is essential that reagents be properly qualified [10,31–33]. Demonstration of manufacturing control is evidenced by strict adherence to standard operating procedures and quality control assessment of manufacturing intermediates, as well as testing of the final cellular preparation.

Because of inherent biological complexity, it is unlikely that a unique biomarker or other single analytical test will be sufficient to permit full characterization of a cellular product. Accordingly, as recommended in "Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy," the FDA asks Sponsors to provide documentation that their testing paradigm developed for the final cell product encompasses a multiparametric approach that may involve biological, biochemical/biophysical, and/or functional characterization [10,33,34]. Therefore, in addition to microbiological testing, tests developed to demonstrate the following should be conceived to determine the degree to which the characteristics of the manufactured cell preparation conform to desired and specified criteria [10,33,34]:

[6] Amendments to Sterility Test Requirements for Biological Products (77 FR 26162).

- identity of the cell product (physical and chemical characteristics identifying the product as being what is designated on the label and distinguishing the product from other products manufactured in the same facility);
- purity (freedom from contaminants including endotoxin, residual reagents, and unintended cell populations); and
- potency/biological activity (the specific ability of the cells, as indicated by appropriate laboratory tests, to effect a given result relevant to the specific indication for use).

This process can be challenging for a number of reasons. For example, the mechanism of action associated with a cell product may be incompletely understood, which may constrain the ability to develop a specific potency assay. Direct assessment of potency for a cellular preparation may not be possible owing to a lack of appropriate in vitro or in vivo assay systems. On Feb. 9–10, 2006, FDA CTGTAC discussed this challenging topic and obtained input on alternative approaches for performing potency assessments of cellular therapy products [29]. A guidance document was issued in Jan. 2011, to provide manufacturers of cellular and gene therapy products with recommendations for developing tests to measure potency [35]. Because process and product development are iterative and will continue throughout the life cycle of the cellular product, continued multiparametric characterization of the cellular product throughout the manufacturing process may aid in identifying critical process steps, establishing relevant specifications and acceptance criteria, and demonstrating comparability after manufacturing changes.

Ensuring the safety of cell products that in and of themselves constitute a regenerative medicine product or that constitute a component of a product requires demonstrated control over each facet of the manufacturing process. This assurance begins with acquisition of the source material and is carried forward through manufacturing and characterization of the final cellular preparation using specified analytical tests based in large measure on the intrinsic biological properties of the cell product.

Xenotransplantation

The success of allogeneic organ transplantation has increased the demand for human cells, tissues, and organs. Scientific advances in the areas of immunology and molecular biology, coupled with the growing worldwide shortage of transplantable organs, have led to increased interest in xenotransplantation. In addition to the potential use of xenotransplantation to address the shortage of human organs for transplantation, there are increasing efforts to use other xenotransplantation products in treating disease. An example of this is the investigational use of encapsulated porcine pancreatic islet cells to treat type 1 diabetes. Along with the promise of xenotransplantation are a number of challenges, including the potential risk for transmission of infectious agents from source animals to patients and the spread of any zoonotic disease to the general public [36]. In addition, the potential exists for recombination or reassortment of source animal infectious agents, such as viruses, with nonpathogenic or endogenous human infectious agents, to form new pathogenic entities. These considerations demonstrate the need to proceed with caution in this area.

The US Public Health Service (PHS) Agencies, including the FDA, the National Institutes of Health (NIH), the CDC, and the Health Resources and Services Administration, have worked together to address the risk of infectious disease transmission, publishing the "PHS Guideline on Infectious Disease Issues in Xenotransplantation" [37]. This Guideline discusses xenotransplantation protocols, animal source, clinical issues, and public health issues. After publication of the PHS Guideline, the FDA published a guidance document entitled "Guidance for Industry: Source Animal, Product, Preclinical and Clinical Issues Concerning the Use of Xenotransplantation Products in Humans" to build on concepts in the PHS Guideline and provide more specific advice regarding xenotransplantation product development and production, and xenotransplantation clinical trials [38].

Xenotransplantation is defined in the PHS Guideline and FDA Guidance as any procedure that involves the transplantation, implantation, or infusion into a human recipient of live cells, tissues, or organs from a nonhuman animal source or human body fluids, cells, tissues, or organs that have had ex vivo contact with live nonhuman animal cells, tissues, or organs.

Examples of investigational xenotransplantation products are:

- transplantation of xenogeneic hearts, kidneys, or pancreatic tissue to treat organ failure, or implantation of neural cells to ameliorate neurological degenerative diseases;
- administration of human cells previously cultured ex vivo with live nonhuman animal, antigen-presenting, or feeder cells; and
- extracorporeal perfusion of a patient's blood or blood component through an intact animal organ or isolated cells contained in a device to treat liver failure.

The use of genetically modified source animals has the potential to overcome physiological and immunologic barriers to successful xenotransplantation as demonstrated in pig to nonhuman primate animal models [39].

When using genetically engineered (GE) animals for xenotransplantation, consultation with the Center for Veterinary Medicine (CVM) before initiating a clinical trial is recommended. GE animals are regulated by CVM under the New Animal Drug provisions of the FD&C Act and its enabling regulations (21 CFR 511 and 514). The requirements and recommendations for producers and developers of GE animals and their products are further clarified in "Guidance for Industry 187: Regulation of Genetically Engineered Animals Containing Heritable Recombinant DNA Constructs." Review teams for xenotransplantation products consist of experts from CBER, CVM, and, when applicable, CDRH.

Medical products that do not contain living cells are not considered to be xenotransplantation products by this definition. Therefore, products that include some common animal-derived components such as collagen, small intestinal submucosa, and heart valves do not automatically fall under this category. When a product does meet the definition, xenotransplantation guidelines are applied as appropriate to the specific product. The FDA encourages any potential sponsor of a xenotransplantation product to familiarize themselves with available documents that can be found on the FDA's website [40].

Gene Therapy

Human gene therapy seeks to modify or manipulate the expression of a gene or to alter the biological properties of living cells for therapeutic use. Products that mediate their effects by transcription or translation of transferred genetic material, or by specifically altering host (human) genetic sequences, are considered gene therapies. Gene therapy products are diverse and include genetically modified viruses (viral vectors), genetically modified microorganisms (e.g., bacteria, fungi), genome-edited/editing products, and ex vivo genetically modified human cells. The FDA regulates human gene therapy products as biological products. The field of gene therapy holds great promise for treating a wide array of illnesses, from genetically inherited diseases such as cystic fibrosis or hemophilia to heart disease, acquired immune deficiency syndrome, graft versus host disease, and cancer. The use of gene therapy in the areas of wound healing, tissue repair, and tissue engineering is also being investigated.

Many of the same regulatory concerns already discussed for human cellular therapies apply to gene therapies, including appropriate qualification of source materials, proper control of the manufacturing process, and sufficient characterization of the final product. There are a number of safety issues associated with gene therapy, however, which are unique to this area. Safety issues specific to gene therapy trials may include generation of replication-competent virus (when using replication-incompetent vectors), vector and/or transgene-associated immune responses, toxicity associated with transgene overexpression, and inadvertent germline transmission of vector. These risks are exemplified by (1) the death of a study subject in 1999 owing to toxicity after administration of high-dose adenovirus vector [41], and (2) genomic integration of retroviral vectors, which has been shown to result in genotoxicity. In the latter case, five children developed leukemia and one died as the direct result of altered gene expression owing to retroviral vector integration [42,43] Detailed recommendations from the FDA regarding what type of CMC information to submit in an early-phase study of gene therapy products are available in the "Guidance for FDA Reviewers and Sponsors: Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Gene Therapy Investigational New Drug Applications (INDs)" [33]. This guidance covers product manufacturing and characterization information (from component qualifications to final formulation procedures), product characterization and release testing (including microbiological testing, identity, purity, potency, as well as other testing), and product stability, giving specifics in these areas that are pertinent to gene therapy.

Special considerations should also be made when conducting preclinical assessment of gene therapy products. For instance, preclinical testing for gene therapy products includes tests designed to determine the biodistribution (i.e., localization and persistence) of the vector in various tissues, as well as assess transgene expression. Additional information on the evaluation of biodistribution in preclinical studies can be found in Section IV.B of the "Guidance for Industry: Preclinical Assessment of Investigational Cellular and Gene Therapy Products" [44]. Clinical trials evaluating gene therapy products may also differ in design from clinical trials for other types of pharmaceutical products because of the distinctive features of these products as well as the target patient populations, outcome measures, and risks involved. Therefore, the FDA has also published advice regarding clinical trial design in the "Guidance for Industry: Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products" [45].

Gene therapies may differ from conventional drugs in that vector and transgene expression may persist for the lifetime of the subject. In these cases, there is a risk for delayed adverse effects. Indeed, the previously mentioned leukemias in a clinical study of gene therapy for the treatment of X-linked severe combined immunodeficiency did not occur until approximately 3–15 years after exposure to the retroviral vector. These events highlight the need for long-term assessment of study subjects receiving gene therapies. The FDA has discussed these issues, noting that the assessment of risk is based on the persistence of vector sequences, the potential for integration into the host genome, and transgene-specific effects. The FDA has published "Guidance for Industry: Gene Therapy Clinical Trials—Observing Subjects for Delayed Adverse Events," which addresses the duration and types of observations to be performed based on the patient population and the risks presented by the gene therapy product [44].

Although regulatory authority for gene therapy clinical investigations rests with FDA, the NIH serves an important complementary role. In addition to funding a number of gene therapy research studies, the NIH provides an important forum for open public deliberation on the scientific, ethical, and legal issues raised by novel FIH gene therapy clinical research applications. This is accomplished through the Recombinant DNA Advisory Committee (RAC), an expert advisory committee to the NIH director [46]. Clinical studies discussed in this forum include studies funded by the NIH, as well as industry-funded studies conducted at clinical sites receiving NIH funding for recombinant DNA research. Also, since the 2013 report by the National Academy of Science and Medicine (previously known as the US Institute of Medicine), NIH-supported clinical investigators have needed to register gene transfer protocols with the NIH Office of Science Policy [47]. In consultation with appropriate regulatory and/or oversight authorities including, IRBs and Institutional Biosafety Committees, the RAC may also identify protocols for public review based on criteria outlined in the 2013 report.

Cell–Scaffold Combination Products

Cell–scaffold combination products often face unique product development challenges because of their inherent complexity. These products often combine metabolically active cells and extracellular matrix or other scaffold components into complex three-dimensional structures, which makes the manufacture, characterization, and study of these products a challenge. Such a complex product that is derived from chemically or physically combining multiple entities cannot be defined solely by the characteristics of the individual components alone. Other factors such as product assembly and the resulting cell–scaffold interactions have critical roles in determining final product characteristics. Furthermore, depending on the intended therapeutic function, these products may be designed to remodel or degrade in vitro during processing in bioreactors and/or in vivo after transplantation during clinical use, thereby precluding complete functionality testing at the time of product release. Packaging, shipping, and shelf life for these dynamic cell–scaffold products are also nontrivial considerations.

As with other products, product safety and efficacy of cell–scaffold products need to be supported by a combination of appropriate in vitro and in vivo preclinical testing. The FDA draws on its extensive experience in regulating mammalian cell products and other tissue-derived products in evaluating product safety and efficacy. Many of the important tests needed for the individual components, such as sterility, mycoplasma, pyrogenicity/endotoxin, scaffold characteristics, adventitious agent testing, cellular viability, identity, and purity, are applicable for the combined product as well. Demonstration of product potency and/or performance is also necessary. The complexity of these products, however, presents challenges for product characterization. Animal models are often used to characterize safety and performance of cell–scaffold products. However, these models have limitations that are confounded by anatomical as well as physiological differences between the animals and humans. Therefore, characterization of these products may require the development of new scientific techniques and assays [48]. In vitro analyses of cell scaffold products may have a critical role in product characterization and evaluation for safety, efficacy, and potency. Nondestructive product testing and rapid characterization tests may also aid in product development. The goals, challenges, and methods of in vitro analyses of cell–scaffold products were discussed at an FDA–National Institute of Standards and Technology (NIST) cosponsored workshop [49]. Challenges and approaches of correlating product characteristics with clinical performance of absorbable medical devices, similar to materials that may be used as cell scaffolds, were discussed at an ASTM International–FDA cosponsored workshop [50].

A critical consideration for developers of cell–scaffold combination products is determining which tests need to be conducted on individual components before assembly and which are most relevant after product assembly. For

example, if undifferentiated cells are directed to differentiate during culture after combination with the scaffold, the individual cellular component will have a phenotype different from that of the cellular component in the final product.

In addition, biocompatibility testing to determine the potential for an unacceptable adverse biological response to the scaffold component is generally most appropriately conducted on the scaffold before combination with the cellular component to prevent convolution of the results. The biocompatibility tests that need to be performed will depend on the type and duration of contact the scaffold will have with the body. Regarding biocompatibility, the FDA issued a guidance document on Jun. 16, 2016 to provide further clarification and updated information regarding the use of International Standard Organization (ISO) 10993-1, "Biological evaluation of medical devices—Part 1: Evaluation and testing within a risk management process" to support applications to the FDA [51].

For many innovative products, such as cell−scaffold combinations used for regenerative medicine, the final product and instructions for use can be expected to undergo iterative modifications over time. Consequently, refinement of the product by the Sponsor and review of product modifications by the FDA will be an ongoing process. It is critical for the Sponsor to have a good understanding of the product and key scientific and/or clinical issues that can affect the safety and efficacy of the product, including the establishment of appropriate manufacturing controls to ensure product quality and consistency. When changes are made in the composition or manufacture of the cell and/or scaffold component of the combination product, it is essential that the Sponsor fully evaluate the impact of such changes to the final product's quality and function.

PRECLINICAL DEVELOPMENT PLAN

The primary goals of the preclinical development program for an investigational device or pharmaceutical product is to establish a scientific rationale for the use of the product in a specific clinical investigation and to demonstrate an acceptable product safety profile. Thus, the conduct of pharmacology and toxicology animal studies is important to identify and characterize possible local and systemic adverse effects. These studies also serve to determine potential safe dose levels in humans and to guide selection of a dose escalation scheme (if applicable) and a clinical monitoring paradigm. For cellular therapy, gene therapy, and cell−scaffold combination products there are frequently additional product-specific safety questions that might need to be addressed before initiation of a clinical trial. For example, for cell−scaffold constructs, there may be safety concerns related to the cells (e.g., inappropriate cell proliferation or differentiation, potential for cell migration from the scaffold) as well as the scaffold (e.g., its ability to perform a specific function at the anatomic site of implantation, degradation properties, structural integrity). These safety concerns may be independent of, or interdependent on, each component of the construct.

As a result of their increasing complexity, many of these products do not have an established paradigm for preclinical evaluation. Sponsors are therefore encouraged to approach the FDA early in their preclinical development program to discuss the scope and design of the preclinical studies that will support the use of their investigational product in a clinical population.

The FDA has held public discussions at Advisory Committee meetings and published Guidance documents that focus on appropriate preclinical testing of cellular therapy, gene therapy, and cell−scaffold combination products. These discussions and publications provide valuable recommendations and considerations for Sponsors as they prepare their preclinical development plan. In addition to various FDA Guidance documents that are referenced throughout this chapter, a list of relevant Advisory Committee meetings can be found in the Advisory Committee Meetings section.

CLINICAL DEVELOPMENT PLAN

The goal of the clinical development program is to establish a product's relative safety and efficacy under specific directions for use in particular diseases or conditions. In the field of regenerative medicine, variability in the product poses unique challenges in clinical trial design and conduct. An additional challenge is the need, with many of these products, to observe their integration into the host over a prolonged period. To facilitate the development of cellular therapy and gene therapy products, the FDA published the "Guidance for Industry: Considerations for the Design of Early-Phase Clinical Trials of Cellular and Gene Therapy Products" [45]. This guidance provides recommendations regarding selected aspects of the design of early-phase clinical trials of cellular therapy and gene therapy products.

However, because of the wide variety of regenerative medicine products and their potential applications, a case-by-case assessment is warranted for the design of each clinical trial. The FDA encourages sponsors to communicate with the FDA throughout product development. Such communications can include formal meetings (e.g., presubmission meeting; end of Phase 2 meeting) and requests (e.g., request for Special Protocol Assessment for products regulated as a biologic, or an Agreement Meeting for products regulated as a device) and informal interactions [20,22,52]. In addition, the FDA encourages IND sponsors to plan ahead by developing and submitting a Target Product Profile (TPP) early in product development. The TPP is an evolving draft of the product labeling and should be revised as clinical development proceeds. Submission of the TPP can facilitate communications between the FDA and the sponsor and enable the FDA to provide more comprehensive advice regarding the overall development program [53].

Food and Drug Administration's Standards Development Program

Since its inception, the development and use of standards have been critical to the mission of the FDA. The use of standards in FDA medical product regulation began with the 1906 Federal Food and Drugs Act. As defined in accordance with the standards of strength, quality, and purity in the US Pharmacopeia and the National Formulary, drugs could not be sold in any other condition unless the specific variations from the applicable standards were plainly stated on the label (Federal Food and Drugs Act, 1906). In current times, federal government agencies, including the FDA, are encouraged when practical to use voluntary consensus standards, whether domestic or international, when performing regulatory activities in lieu of government-unique standards that are developed by the government for its own uses. Standard-setting activities include the development of performance characteristics, testing methodology, manufacturing practices, product standards, scientific protocols, compliance criteria, ingredient specifications, labeling, or other technical or policy criteria.

As with guidance document development, in which Good Guidance Practices describe the FDA's procedures for developing and using guidance documents, specific regulations describe FDA participation in outside standard-setting activities. Regulations governing this participation can be found in 21 CFR 10.95. In addition, the FDA's Staff Manual Guide 9100.1 establishes agency-wide policies and procedures related to standards management activities to ensure a unified approach to standards within the FDA. Constructive FDA participation in organizations responsible for developing standards applicable to the products regulated by the agency is considered essential.

The FDAMA of 1997 provides for the recognition of national and international standards in medical device reviews for IDEs, HDEs, PMAs, and 510(k)s [54]. A "recognized consensus standard" is a consensus standard that the FDA has evaluated and recognized, in full or in part, for use in satisfying a regulatory requirement and that the FDA has published in a Federal Register notice. A "consensus standard" is a standard developed by a private sector standards body using an open and transparent consensus process. Conformance with recognized consensus standards is strictly voluntary for a medical device manufacturer, who may choose either to conform to applicable recognized standards or to address relevant issues in another manner. Standards may also be used in support of non-device applications when appropriate and not in conflict with an FDA regulation or Guidance. Lists of recognized standards, Guidance Documents, and Standard Operating Policies and Procedures can be found on the FDA's website.

The scientific and manufacturing novelty of many regenerative medicine products presents unique challenges for meeting regulatory requirements with respect to product testing, developing performance characteristics, testing methodologies, scientific protocols, product standards, ingredient specifications, and compliance criteria. Increased development and use of standardized analytical methods and metrics have the potential to facilitate product characterization and reduce time to market by leveraging industry and government efforts. CDRH and CBER actively engage with Standards Development Organizations (SDOs) such as ASTM International and the ISO. CDRH and CBER staff attend standards meetings and workshops and participate in developing the standards and formally voting on the publication of standards. ASTM Committee F04 on Medical and Surgical Materials and Devices Division IV is actively engaged in developing standards for tissue engineered medical products (TEMPs). F04 Division IV consists of six subcommittees: (1) Classification and Terminology, (2) Biomaterials and Biomolecules, (3) Cells and Tissue Engineered Constructs, (4) Assessment, (5) Adventitious Agent Safety, and (6) Cell Signaling. The ASTM TEMPs group has developed more than 25 published standards, including standard guides, standard practices, and test methods, and has approximately 30 draft standards under preparation. The first of these standards were developed for substrates, biomaterials, natural materials such as collagen, alginate, and chitosan, terminology, cells and cell processing, bone morphogenetic protein, assessment of adventitious agents, and test methods for

characterizing biomaterials. These standards are reviewed on a regular basis by the appropriate ASTM subcommittee (SC) to ensure that the standards reflect current scientific knowledge. The FDA liaisons elected to represent the Agency on each of the six subcommittees review the standards to ensure that standards developed do not conflict with current FDA regulatory practices. Some examples of approved standards with which the TEMPs group was involved include standards for characterizing and testing biomaterial scaffolds, quantifying cell viability within biomaterial scaffolds, and repairing articular cartilage in vivo.

Another SDO with which FDA is involved is ISO, a nongovernmental international organization that develops consensus standards in collaboration with both the private and public sector. Standards for regenerative medicine/tissue engineering products are developed in Technical Committee (TC) 150 SC 7, Tissue Engineered Medical Devices, and in TC 194, Biological Evaluation of Medical Devices. Within TC 150, SC 7 are two active Working Groups (WGs): WG 1 Management of Risk, and WG 3 Tissue-engineered medical products for skeletal tissues. TC 194 SC 01 is responsible for Tissue Product Safety, and within SC 01 are four WGs: WG 01 Risk Assessment, Terminology, and Global Aspects, WG 02 Sourcing Controls, Collection, and Handling, WG 03 Elimination and/or Activation of Viruses and Transmissible Spongiform Encephalitis (TSE) Agents, and WG 04 TSE Elimination. ISO standards of particular interest to regenerative medicine products are those in the 10993 series (10993-1−20). Additional ISO standards-setting activities for regenerative medicine/tissue engineering products take place in TC 276, Biotechnology, WG 01 Terminology, WG 02 biobanks and bioresources, WG 03 analytical methods, WG 04 bioprocessing, and WG 05 data processing and integration. Standards are available through ISO's website: http://www.iso.org.

There are many benefits to standards development, adoption, and use. Participation in standards development activities benefits the regenerative medicine community in that these activities can facilitate the development and maintenance of guidance for industry, address issues not addressed in FDA Guidance documents, facilitate product design, and lead to international harmonization. From a business perspective, participation in standards development activities can provide a competitive advantage by influencing technical contents, reduce the cost of production by improving factory flexibility and supply chain management, foster innovation and support research and development, and increase trade by shortening the time between concept and global availability. Standards can be used by developers of regenerative medicine products to meet regulatory expectations. In turn, the benefit to the regenerative medicine community helps the public by providing products that are more thoroughly characterized with a greater understanding of the conditions of use of the products. Thus, the FDA has a critical role in providing support for standards development activities.

ADVISORY COMMITTEE MEETINGS

As mentioned in the Meetings With Industry, Professional Groups, and Sponsors section, because of the diversity of innovative technology evaluated by FDA review staff, the FDA makes use of scientific Advisory Committees or Panels (for medical devices) to complement its internal review process. These advisors provide independent, professional expertise and technical assistance to contribute to scientific regulatory decision-making processes. Outside experts can be asked to review data or make recommendations about study designs across a product or clinical area; outside advisors can also be helpful at earlier stages of product development. Expertise on the advisory committee often includes scientific, statistical, and clinical experts, as well as consumer representation, patient advocates, and industry participation. Most meetings are public, and there is an opportunity for public participation in the form of public comment.

There are 34 Advisory Committees as well as a number of SCs and one DHHS Committee administered by CBER. The areas of responsibility for the Panels and committees are divided along medical specialty areas. The Advisory Committee for cellular, tissue, and gene therapy products, as mentioned earlier, is the CTGTAC. This committee has discussed a number of topics that are of potential interest to product developers in the regenerative medicine area, including:

- Hematopoietic stem cells for hematopoietic reconstitution (Feb. 2003),
- Allogeneic islet cell therapy for diabetes (Oct. 2003),
- Somatic cell cardiac therapies (Mar. 2004),
- Cellular products for joint surface repair (Mar. 2005),
- Potency measures for cell, tissue, and gene therapies (Feb. 2006),

- Cellular therapies derived from human embryonic stem cells—considerations for preclinical safety testing and patient monitoring (Apr. 2008),
- Animal models for porcine xenotransplantation products intended to treat type 1 diabetes or acute liver failure (May 2009),
- Clinical issues related to FDA draft guidance "Preparation of IDEs and INDs for Products Intended to Repair or Replace Knee Cartilage" (May, 2009),
- Testing of replication competent retrovirus/lentivirus in retroviral and lentiviral vector-based gene therapy products (Nov. 2010),
- Cellular and gene therapy products for the treatment of retinal disorders (Jun. 2011), and
- Oocyte modification in assisted reproduction for the prevention of transmission of mitochondrial disease or treatment of infertility (Feb. 2014).

The presentations for each topic, as well as transcripts of each discussion, are available on the FDA's website referenced at the end of this chapter [29].

The Medical Devices Advisory Committee consists of 18 Panels that cover the medical specialty areas. Panel meetings are held regularly to discuss specific products. A few examples of past Panel meetings are:

- General and Plastic Surgery Panel regarding dermal fillers (Nov. 2008),
- Orthopaedic and Rehabilitation Devices Panel regarding a bone putty (Mar. 2009),
- Ophthalmic Devices Panel regarding a visual prosthetic device (Mar. 2009),
- Neurological Devices Panel regarding a spinal surgery sealant (May 2009),
- Ear, Nose, and Throat Devices Panel regarding an implantable hearing system (Dec. 2009),
- Orthopedic and Rehabilitation Devices Panel regarding metal-on-metal hip arthroplasty systems (Jun. 2012),
- Circulatory Systems Devices Panel regarding an atrial appendage device (Oct. 2014), and
- General and Plastic Surgery Devices Panel regarding wound care products combined with antimicrobials or other drugs (Sep. 2016).

A complete list of upcoming Medical Devices Advisory Committee Panel meetings and searchable archive of past meetings with agendas and materials can be found on the FDA's website [55].

FOOD AND DRUG ADMINISTRATION RESEARCH AND CRITICAL PATH SCIENCE

The FDA recognizes the complexity of the scientific issues related to regenerative medicine products. FDA research laboratories have an important role in ensuring that the agency stays abreast of the rapid changes and developments affecting the entire field as well as addressing specific regulatory science questions. In 1992, CBER researchers began systematic efforts to uncover mechanisms that control the behavior of cells subjected to various environmental stimuli, particularly those encountered during normal wound healing, regeneration, and prenatal development. Those studies led to the discovery of several growth factors and feedback mechanisms that control these pathways [56–63]. Other research addressed interactions between hematopoietic and mesenchymal cell lineages both in vitro and in vivo [64,65]. The success of those efforts led to the recruitment of additional investigators in these areas. In 2004, the FDA introduced the Critical Path Initiative to identify and support research priorities that are expected to advance innovation in medical products. The Critical Path Opportunities List "presents specific opportunities that, if implemented, can help speed the development and approval of medical products" [66]; it is available on the FDA's website. A number of the research topics on the Critical Path Opportunities List have applications to regenerative medicine, such as developing characterization tools for cell therapy and tissue engineering, biomarkers for cardiovascular disease, and advanced imaging technologies. FDA laboratories are actively engaged in these and other research questions that will facilitate the advancement of the field of regenerative medicine.

A priority of the Critical Path Initiative is updating and modernizing techniques, to ensure that the agency and the research community have the tools necessary to bring safe and effective products to market. Efforts include promoting collaborations spanning multiple centers and regulatory jurisdictions across the FDA as well as among the FDA and other relevant organizations (e.g., other agencies, academic organizations, regulated industry). Developing research collaborations and the requisite infrastructure to support those and other efforts will facilitate review of combination products, which are often seen in regenerative medicine.

An example of collaborative work across FDA laboratories is a multiinvestigator project at CBER using a battery of state-of-the-art analytic techniques chosen to provide complementary data on cell state and seeking to develop

new biomarkers for cell therapies. The project aims to characterize mesenchymal stromal cells from a number of perspectives, including genetic stability, proteomic and phosphoproteomic analysis, microRNA analysis, messenger RNA profiling by microarray and quantitative polymerase chain reaction analysis, chromatin immunoprecipitation, and examination of the potential for cells to mature and contribute to the formation of organs and tissues. Furthermore, the study will look for molecular differences between cells from early versus late passage numbers. Importantly, the same cells that go through this panel of tests will be implanted in a mouse model of hind-limb ischemia, allowing for the correlation of product characterization data with the in vivo outcome with regard to localization, differentiation, and functionality. This FDA research project may yield information that will be useful for product characterization, in-process testing, lot release criteria, developing comparability and stability protocols, and predicting cell fate and function after receiving a cell therapy.

A major issue associated with the clinical use of cellular therapies is predicting what happens to the cells after administration. Another FDA Critical Path research study at CBER will advance cell therapy by developing methods for in vivo tracking and imaging of neural stem cells (NSCs) after transplantation. NSCs from adult, fetal, and embryonic sources have been proposed as treatments for degenerative conditions such as Parkinson disease and for the repair of tissues damaged by stroke and spinal cord injury. Magnetic resonance imaging and single photon emission computed tomography are being used to determine cellular location and the persistence of engraftment qualitatively and quantitatively. The goal of this project is to develop methods for evaluating biomarkers that may be predictive of NSC function.

The FDA's research projects often involve collaborations with other federal and academic partners to employ new technologies to address regulatory science questions. For example, a collaboration between FDA and the NIST is using automated microscopy to characterize the differentiation of mesenchymal stem cells (MSCs). The goal is to improve the safety of MSC products by developing robust assays that can be used for in-process and lot release testing.

FDA Critical Path research also addresses some of the challenges faced in both product development and product evaluation. For example, after the observation of unexpected toxicity of adenoviral vector gene therapy in a clinical trial, CBER research provided insight into how adenovirus vectors cause toxicity and developed an animal model for gene therapy in the context of preexisting liver disease [67]. CBER researchers and regulators also worked with a consortium from industry and academia to develop reference material for adenoviral vector particles [68]. FDA research is ongoing to understand the nature of toxicity of systemically delivered adenovirus and mechanisms for vector clearance to improve the safety of gene therapy trials [69].

Some FDA laboratories are engaged in research projects related to tissue. CDRH scientists are studying the effects of mechanical and electrical stimulation on cardiac cell cultures and how the parameters modulate cellular physiology. A CBER–CDRH collaboration is examining the relationship between encapsulation of chondrocytes in a scaffold material, with and without mechanical stress, on the status of several signaling pathways. Another intercenter collaborative research project between CDRH and CDER conducts a comprehensive translational assessment of human induced pluripotent stem cell-derived cardiomyocytes to evaluate drug-induced arrhythmias [70] and to predict individual patient response to drugs by using patient-specific stem cells collected in an FDA-sponsored clinical trial [71]. A Critical Path collaborative research project with partnerships outside the US government involves CDRH, CBER, the University of Maryland, the University of Akron, and Tufts University, and evaluates the accuracy and reproducibility of three-dimensional (3D) printed scaffolds containing cells. This project assesses the material and biological properties of the printed scaffolds in the presence or absence of cells on printing accuracy.

The FDA's interest in 3D printing, also known as additive manufacturing, is in part because it is driving the innovation of medical products, including in the area of biological and tissue engineered products for regenerative medicine purposes. 3D printing involves the layer-by-layer deposition of material to produce a 3D part from a digital design file. With the increase in use of 3D printing and the uncertainty of how the technology can affect the safety and effectiveness of the products, interest in 3D printing has increased at the FDA and led to the formation of the Additive Manufacturing Working Group. The WG held a public workshop on Oct. 8–9, 2014, to obtain input from stakeholders, entitled "Additive Manufacturing of Medical Devices: An Interactive Discussion on the Technical Considerations of 3D Printing" [72]. The workshop aimed to bring together experts in the field to discuss with the FDA the current state of the art of 3D printing, and included discussions on 3D printing of medical devices, biologics, and pharmaceuticals. Some of the considerations discussed during the public workshop are presented in FDA web content and documents on 3D printed devices [73] and a technical review [74] These documents primarily focus on medical devices, with important considerations for evaluating 3D printed devices including the following: the effect of build orientation and location on final device performance, including mechanical and physical

properties; process validation of the 3D printing systems to ensure consistency between print jobs; sterilization and removal of residual materials; and characterization of material properties before and after printing.

In the case of printing biological products, also known as bioprinting, applications may include printing tissue scaffolds, constructs containing living cells [75], and even small tissue [76] and organ structures [77]. 3D printing may offer an approach to make a medical product with a complex architecture, such as porous structures or internal lattice structures. In addition, cells and biomaterials could potentially be printed simultaneously with more precise spatial control to produce constructs with desired properties. Although the FDA has experience regulating 3D printed medical products [74] no FDA-approved or cleared biological products incorporate 3D printing. These types of products will typically be regulated along the same pathways as non-3D printed products. However, because of the complexity of 3D printed biologics, there may be some additional technical considerations for sponsors to take into account and evaluate compared with non−3D printed products. Some of these considerations may include, but are not limited to, the printing parameters and consistency, material selection, finishing steps, biocompatibility, mechanical and physicochemical properties, and the biological function of the finished product. Known applications of bioprinting technology used for research purposes include skin [76], cartilage [78], bone [79], nerve [80], and blood vessels [81].

With accelerating advancement in this area, the FDA continues to communicate with the public regarding considerations for 3D printed medical products. Through ongoing research collaborations, and discussions with stakeholders, the FDA is committed to fostering safe and effective innovation in the area of 3D printed medical products.

With support through the Critical Path Initiative, FDA research laboratories provide an important source of in-house expertise in regenerative medicine and other cutting-edge technologies and research areas. Although by no means exhaustive, the examples provided here demonstrate the diverse range of topics under investigation at the FDA. Especially in consideration of the rapid change and development of the regenerative medicine field, Critical Path research efforts ensure that the FDA stays abreast of current innovations. The FDA's research programs provide an important source of the latest science to inform the regulatory process and bring safe regenerative medicine products to market.

OTHER COORDINATION EFFORTS

Because of the highly interdisciplinary nature of regenerative medicine, the FDA recognizes the need to build collaborative efforts to review products successfully in this area. The FDA is therefore a partner in the Multi-Agency Tissue Engineering Science (MATES) Interagency Working Group. This partnership, which spans more than a dozen federal agencies, is designed to provide a forum to facilitate communication and coordination across the government regarding activities in tissue engineering and regenerative medicine. The full strategic plan can be found at the MATES website [82].

In another example of interagency interaction, collaboration, and coordination, the FDA has established Memoranda of Understanding (MOU) agreements with two different NIH institutes that involve FDA scientific review staff and NIH extramural research program officers. The MOUs cosigned by the National Institute of Neurological Disorders and Stroke and the National Heart, Lung and Blood Institute incorporate safeguards to protect from disclosure shared, nonpublic information such as trade secrets and confidential commercial information, identities of study participants and other personal information, privileged and/or predecisional agency information, research proposals, progress reports, and/or unpublished data or information protected for national security reasons. Under the MOU agreements, participants are able to hold unfettered discussions and exchange information that enables the respective agencies to maintain currency with respect to ongoing scientific activities that could affect regenerative medicine from both the laboratory and clinical research perspectives. Interagency MOU interactions identify gaps in knowledge related to the state of available scientific information and familiarity with FDA regulatory expectations. This, in turn, contributes to identification of promising basic research with the potential for clinical translation.

There are also efforts to promote collaboration within the agency. For example, the FDA Commissioner's Fellowship Program (CFP) is facilitating a collaboration related to the regulation of regenerative medicine across the FDA's product jurisdictions. The CFP was established in 2008 to attract new talent to the agency while providing an opportunity for those to learn about FDA regulatory science for future careers outside the agency. Within its annual cohort of fellows, a regenerative medicine fellowship program has been established. Because many regenerative medicine products involve combining biologics and device technology, regenerative medicine fellows work across both CBER and CDRH to facilitate cross-agency collaboration and conduct research projects related to the regulation of regenerative medicine products.

Interaction between the FDA and the scientific and regulated communities is an important area of collaboration. Workshops are one such example, including the previously mentioned FDA-NIST cosponsored session regarding cell–scaffold products. Workshops provide valuable opportunities for the agency to receive input from outside scientific experts and other stakeholders. An additional common example of this type of activity is the liaison meeting, in which the FDA directly engages in dialog with professional societies (e.g., International Society for Stem Cell Research, American Association of Blood Banks, American Association of Tissue Banks, International Society for Cellular Therapy) regarding scientific or regulatory issues related to a certain research area. The FDA is a founding member of the National Academy of Medicine (NAM) Forum on Regenerative Medicine. This forum, which is similar in structure and function to NAM's longstanding Drug Forum, provides a neutral convening mechanism for interested parties from academia, industry, government, patient/provider organizations, regulators, foundations, and others to discuss difficult issues facing the application of, and opportunities for, regenerative medicine. The Kidney Health Initiative, an existing public–private partnership that includes the FDA, has begun a roadmapping project to develop renal replacement therapies. CBER and CDRH have a leading role in this effort. All of these activities ensure that the FDA receives continuous input on the latest scientific discoveries to inform the regulation of safe products.

CONCLUSION

The field of regenerative medicine is exciting, with scientific advances leading to the promise of future therapies for current unmet medical needs for patients. The FDA regulatory approach to the evaluation of medical products includes an ongoing assessment of how the science of those products informs regulatory policy. To meet the needs of the challenging array of products that are on the horizon, the FDA intends to continue the current dialog with the scientific community and product Sponsors as part of its mission to develop science-based regulatory review policies that are robust and predictable.

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CHAPTER

78

Regenerative Medicine Manufacturing—Challenges and Opportunities

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WHY REGENERATIVE MEDICINE MANUFACTURING?

Why regenerative medicine manufacturing? The answer is simple. For regenerative medicine-based technologies and products to become the next standard of care, advances are needed in manufacturing processes. This chapter will highlight the current challenges facing regenerative medicine and list what the primary challenges are that are hindering widespread adoption. We will then consider some of the challenges in lack of standards and lack of definition of quality, as well as some of the technical challenges. After highlighting these challenges, we will delve into the opportunities that exist in regenerative medicine manufacturing. These opportunities exist in areas of regulatory and standard setting and we will cover some of work currently being done in these areas. There are also opportunities for new technologies and we will cover some of the advances being made for scale-up, reducing costs, quality control systems, automation, modular plug and play systems, and bioprinting. Next we will consider regenerative medicine manufacturing systems of the future. While these systems do not currently exist in full form today we believe these systems will be part of every major clinical regenerative medicine program in the next 20 years. We will conclude this chapter by reviewing the global landscape focusing on technical societies and domestic and international efforts.

CURRENT CHALLENGES AND OPPORTUNITIES IN REGENERATIVE MEDICINE MANUFACTURING

Primary Challenges for Widespread Adoption

The primary challenges for widespread adoption of regenerative medicine-based technologies have been reviewed extensively [1–7]. Table 78.1 highlights some of these challenges. While the science of regenerative medicine continues to progress, for widespread adoption we must solve several roadblocks related to the manufacturing aspect of these treatments. Currently, high cost, quality, and logistics issues threaten the promise of bringing regenerative medicine therapies to those in need. The lack of efficient, controllable manufacturing processes makes cost and quality difficult to achieve. Moreover, Food and Drug Administration (FDA) approval procedures make continuous improvement far more difficult and costly.

More efficient, cost-effective manufacturing will require advances in automation and control to remove human work and reduce variability where possible, because differences in procedures induce increased variability. Improved biosensors will also be needed to control quality and consistency, which will also drive down cost.

Cell expansion is an example of an area, common to all therapies, in need of cost-effective scale-up. Progress is being made in the development of scalable suspension cultures that can generate billions of human cells using

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TABLE 78.1 Current Challenges in Regenerative Medicine Manufacturing

Need for scale-up (e.g., expand to billions of cells)
High costs of manufacturing regenerative medicine product
Lack of sufficient quality control systems for in-line sensing
Lack of automation
Lack of closed and modular systems
Lack of standards for regenerative medicine

single-use bioreactors [8,9]. This necessitates advances in bioreactor design, as well as sensors to meet the large quantities of cells for a given product. Additionally, it should reduce cost and make these therapies more widely available [10].

Lack of Standards

Regenerative medicine covers a broad range of therapies as it manipulates genes, cells, and tissues to repair or replace diseased, damaged, or missing organs. This may include bone, skin, as well as other cells and tissues. The transition from laboratory to clinic has been slow due to the inherent complexity of the treatments and a lack of standards and standardization that complicates manufacturing thereby increasing cost. Many traditional measurements of efficacy, potency, purity, and quality that work with traditional pharmaceutical manufacturing may not be sufficient for regenerative medicine treatments.

The National Institute of Standards and Technology (NIST) has developed a regenerative medicine and advanced therapies laboratory program that advances cell therapy, gene therapy, and tissue engineering by developing measurement infrastructure, including enabling tools, reference materials, methods and protocols, and bioinformatics and modeling tools [11]. NIST also has a regenerative medicine biomanufacturing program that is seeking to develop measurement solutions, serve as a neutral ground for the discussion of underpinning measurements and other manufacturing needs, and also lead and contribute to the development of standards [12]. NIST has also entered into a memorandum of understanding with the Standards Coordinating Body for Gene, Cell, and Regenerative Medicines and Cell-Based Drug Discovery to advance the field by developing consensus standards.

ASTM International and the International Organization for Standardization (ISO) have a partnership in additive manufacturing where they have jointly released the Additive Manufacturing Standards Development Structure [13]. This provides a framework for meeting the needs for new standards in areas including regenerative medicine. ASTM also has many technical committees that serve as working groups to develop new standards in areas such as additive manufacturing (Committee F42) and medical and surgical materials and devices (Committee F04). ISO is another standard setting organization that brings together technical experts from around the world to develop international standards. For instance, there are ISO standards currently available for tissue-engineered medical products (ISO 19090:2018) and quality management systems (ISO 90001:2015 and ISO 13485:2016).

For cell therapies, standards for imaging tools and protocols for cell characterization (identification, quantity, and function) are needed, as well as reference materials for fluorescence imaging and flow cytometry. For tissue engineering standards for scaffold design, structure and properties are needed as are standards for the computational models required. FDA has released guidance for additive manufactured devices that are implanted [14]. While these are not yet approved and some may not apply to scaffolds, they illustrate the complexity of standards development for future manufacturing systems and the need to incorporate them into process design. These are summarized in Table 78.2. In addition to standards, the manufacture of regenerative medicine products needs standardization of procedures and protocols to minimize variability, thereby increasing quality and decreasing costs.

Logistics

Currently, cells are preserved and stored at cryogenic temperatures. This impacts handling procedures, cost, efficacy, safety, equipment requirements, retrieval, and effects on the regenerative medicine product. In addition, the ability to distribute product to point-of-use locations is constrained by the number of cryogenic containers.

TABLE 78.2 Summary of Recent Food and Drug Administration (FDA) Guidelines for Additive Manufactured Devices

Area	Components
Overall device design	Comparison of desired feature sizes and tolerances to process
Patient-matched device design	Effects of imaging, software interacting with design models, complex design file conversion, cybersecurity and personally identifiable information
Software workflow	File format conversions, digital device design to physical device, build volume placement, addition of support material, slicing, build paths, machine parameters and environmental conditions, automated software validation
Material controls	Starting material, material reuse
Postprocessing	Full documentation
Process validation and acceptance activities	Process validation, revalidation, acceptance procedures, test coupons
Quality data	Analysis of sources of quality data to identify existing and potential causes of nonconforming product
Device testing	Description, mechanical testing, material characterization, residue removal and sterilization
Labeling	Recommended for Additive Manufactured (AM) devices that are patient matched, may include patient identifier, use, final design iteration or version used to produce the device

Sensors and Quality Control Systems

There are significant challenges with the development of sensors and quality control systems for regenerative medicine manufacturing, although they differ between cell expansion, tissue, and organs. Product loss or slow growth for cell-based therapy batches may slow testing or attainment of full expansion. For cells, tissue and organ sensors or sensor suites are needed to properly control processes and assure quality.

Bioprinting

While there a many bioprinters on the market, none meet the proposed FDA guidelines and can be used for human patients. As summarized in Table 78.2, there are many issues covered in the FDA proposed guidelines and no currently marketed bioprinter meets these. It is important to note that issues involving hardware design, software, material control, and validation will make this challenging.

Scale-Up and Automation

Benchtop research does not easily scale up for production for a number of reasons. Since there is little standardization of processes causing poor reproducibility the translation to production is difficult. Also, it is time consuming and expensive to change protocols once approved by the FDA. Standardized protocols, whether for manual or automated manufacturing, will help reduce variability and enable scale-up. However, the human element adds uncertainty and cost. Therefore automation for autologous products is crucial for widespread adoption. Currently, there is no marketed, fully integrated, modular manufacturing system.

Collectively, we have highlighted some of the opportunities in regenerative medicine manufacturing and listed them in Table 78.3. We mention that there can be technologies developed that can assist with scaling-up processes such as using single-use technologies [15]. There is also an opportunity for developing solutions for reducing costs, which could include multiple solutions discussed further in this perspective article [10]. Other opportunities exist with developing in-line sensing systems, automating processes through integration of robotics and artificial intelligence, and developing plug and play infrastructures to accommodate different manufacturing processes. A final set of opportunities exists in the development of standards and ensuring seamless integration of these new regenerative medicine-based products with regulatory pathways. The FDA has published two new draft guidance documents that are intended to assist with bringing innovative, safe, and effective products to patients as efficiently as possible. The first guidance document is called "Expedited Programs for Regenerative Medicine Therapies for Serious Conditions." The second document is called "Evaluation of Devices Used with Regenerative Medicine Advanced

TABLE 78.3 Current Opportunities in Regenerative Medicine Manufacturing

Develop technologies for scale-up (e.g., single-use technologies)
Develop solutions for reducing cost (e.g., defined media)
Develop solutions for quality control systems (e.g., microfabricated biosensor technologies for in-line sensing)
Develop technologies that can automate processes (e.g., integration of robotics and artificial intelligence into manufacturing processes)
Develop solutions for modular systems (e.g., plug and play infrastructure to accommodate different modules)
Current ASTM and ISO efforts for developing standards (e.g., ISO 19090:2018 for tissue-engineered medical products)
FDA guidance documents for advancing regenerative medicine-based products (e.g., Technical Considerations for Additive Manufactured Medical Devices)

Therapies." The first guidance document describes the Fast Track designation and breakthrough Therapy designation, which are available for regenerative medicine therapies. It also describes requirements for the new Regenerative Medicine Advanced Therapy (RMAT) designation program, which was created by the 21st Century Cures Act. The second draft guidance document on devices provides the FDA's current thoughts on evaluating devices used in the recovery, isolation, and delivery of RMATs.

ENVISIONED REGENERATIVE MEDICINE MANUFACTURING SYSTEMS OF THE FUTURE

Envisioned regenerative medicine manufacturing systems of the future will enable these technologies, therapies, and products to be commercialized and made widely available. Table 78.4 illustrates some of these envisioned system areas, their system attributes, and companies of interest that have some of the capabilities needed to build these systems. Fig. 78.1 captures some of the envisioned manufacturing systems of the future that could bioengineer new tissues and organs by integrating a clinical-grade 3D bioprinting approach into the manufacturing process. Next we will cover some of the system attributes we believe will be critical for these advanced regenerative medicine manufacturing systems.

Standardization will be critical for future regenerative manufacturing systems due to the inherent variability and need to make procedures more consistent within and between manufacturing sites. Both manual and automated manufacture will rely on adherence to standards and standardization. NIST is working to establish standards for cell measurement, live cell imaging, quantitative flow cytometry, and bioprinting. One concern is that measurements of efficacy, potency, purity, and quality that are used for pharmaceuticals may be insufficient for regenerative medicine. Moreover, standardized procedures for cell expansion, size of biopsies, and other critical operations are needed for manual and automated manufacture.

Fully integrated, modular, and automated manufacturing systems are critical and this will help to drive standardization since automation will depend, to a certain extent, on this. Such a system would utilize closed pods or cassettes for all processing to remove the possibility of contamination. This "plug and play" design philosophy would also lend flexibility to these manufacturing systems allowing them to produce a wide array of regenerative medicine products for individuals. An integrated modular robotic system with closed pods could have the ability to exploit standard processes, including cell harvesting, cell processing, nutrient addition, tissue digestion, incubation, imaging and characterization, tissue banking, quality control, and preservation prior to use or shipping.

The collection of data, scheduling, and control of such a system will be complex and require new sensors and smart controls. The use of machine learning to sense quality characteristics and remove human visual perceptions (and biases) will be needed. Control for processes that can be highly variable may take weeks and must be done in a way that gets needed cells, tissue, and organs to critically ill patients reliably. Smart manufacturing refers to

TABLE 78.4 Envisioned Manufacturing Systems of the Future

Future System Areas	System Attributes
Fully integrated/modular/closed/sterile/automated manufacturing systems	• Closed, integrated purification, formulation and vial fill • Seamless media to bioreactor/cell culture vessel transition • Cell concentration standard method to maintain viability/potency • Customizable modules for expansion and cell retrieval • Automated cell handling in a fully controlled aseptic environment. Scale-up for mass production • Passaging and layering multiple types of cells in a closed system • Automatic monitoring of glucose utilization/lactose production to adjust nutrient supply for continuous feed • Off-the-shelf closed systems that can be easily upscaled
Xeno-free defined media systems	• Synthetic serum for human immune cells and mesenchymal stem cells first. Following its success it can be extended to other tissue sources in the human body • Universal "basal" media • Synthetic, defined serum substitute "panel"
Supply chain and logistic systems	• Platform technologies for shipping human stem cells and mesenchymal stem cells should be initially tested • Further development of formulations and methods for extending liquid storage stability • Further development and optimization of formulations and methods for freeze drying
Biosensing systems	• 3D printer for engineered tissues • Microfabrication of biosensors • Better oxygen and glucose monitoring sensors • Improved material for adherent cells to maximize cell seeding and harvesting
Nondestructive quality control systems	• One area is lactate and ammonia management • In-line measurement of cell density • Real-time cell "state"/phenotype monitoring • Data capture and mining/correlation • Microscope with software for recognizing and quantifying cellular structures • Disposable sensors or built-in sensors for growth factor levels in addition to pH, osmolarity, O_2, and CO_2. Glucose, ammonia, and K^+ would be great as well • There will not be a path to a universal solution—too divergent. Pick either most universal quality control specification/parameter (most therapy agnostic) or highest impact upcoming therapy—propose and prototype a solution
Automated and closed patient-specific systems	• Integrated, standardized disposables for product types • Processes that are patient specific and would involve modeling the patient's anatomy • Easily adaptable semiuniversal automated system • Semiuniversal disposables • Automated injector with detailed process control • Novel single-use sensors/sensing approaches for noninvasive monitor/control of process parameters and detection of microbial contamination • Disposable single-use bioreactors that will support all steps from seeding through harvest • Instrumentation that can provide CO_2 and temperature control without traditional incubators
Cell and tissue expansion systems	• Determine if same system could be scaled to fit both small- and large-scale needs • In-line cell "state"/phenotype monitoring • Scalable bioreactor technology • Novel single-use format for high-density cell culture (adaptable adherent or suspension, or tissue constructs) • Design for scale-out strategy for autologous therapies

FIGURE 78.1 Envisioned future manufacturing systems: illustrated here is an envisioned future manufacturing system where 3D bioprinting will be incorporated into an end-to-end modular manufacturing system that can produce at scale bioengineered tissues and organs.

"fully-integrated, collaborative manufacturing systems that respond in real time to meet changing demands and conditions" [16]. It has the potential to use data and models to control such complex systems. Another aspect of an integrated automated manufacturing system is the ability to share information. Blockchains, a computer science construct that uses a distributed recording and storage of transaction records in a manner that safeguards patient confidential information, can be used to link information through the process to understand cause and effect and better control production.

Xeno-free defined media systems will be able to support a large panel of clinically relevant cell types that will be needed for cell and gene therapies, as well as tissue engineering applications. These systems will greatly accelerate product development times, as well as reduce costs and variability by removing serums and recombinant proteins and replacing them with defined biochemical substitutes. The RegenMed Development Organization is currently using a consortium-based model to develop a platform technology that seeks to build a universal media system similar to the one we envision here to support clinical cell manufacturing. Efforts like these will greatly accelerate the field.

Biosensing systems will be integrated into regenerative medicine manufacturing processes to monitor the viability, phenotypic characteristics, biomechanical characteristics, and physiologic responses of cells, scaffolds, organoids, organoid systems (e.g., body-on-a-chip personalized medicine systems), tissues, and bioengineered organs. These biosensing systems will be microfabricated and allow improved monitoring of oxygen, glucose, metabolites, pH, temperature, and many additional attributes that can be customized based on the cellular and tissue-specific systems being engineered and manufactured. These biosensing systems can also be integrated into 3D bioprinting systems to ensure the integrity of starting materials (e.g., cells, biomaterials, bioinks, etc.) and confirm the viability, phenotypic characteristics, biomechanical characteristics, and physiological responses from 3D bioprinted organoids, tissues, and organs.

Nondestructive quality control systems will provide in-line measurement of specific quality control attributes needed in regenerative medicine manufacturing processes of the future. One area of need will be improved monitoring of lactate and ammonia management, as well as other biochemical markers that provide important information to maintain the viability of cellular and tissue systems. These in-line measurements will capture data in real time and have artificial intelligence systems to make correlations and predictions on safety and quality attributes of the current and final clinical product that will also save time and money by only advancing products with a high probability of possessing final safety and quality attributes needed for the manufactured clinical product.

Cell and tissue expansion systems will be adaptable to both small- and large-scale needs. The miniaturized systems will enable autologous patient-specific therapies at the bedside, while the large-scale systems will provide a pathway for allogeneic therapies to be manufactured at industrialized scale for widespread use. Within these two systems, in-line cell "state" or phenotype monitoring will be critical to ensure safety and quality attributes are maintained throughout the manufacturing process. There will also be opportunities for novel single-use formats for high-density cell cultures.

Supply chain and logistic systems must be used carefully when considering the expansion of regenerative medicine therapies. Current technologies are expensive as companies must buy and maintain an appropriate number of cryogenic storage containers for shipment. Otherwise, timely shipment and product quality cannot be guaranteed.

Current thinking and research seeks novel cell preservation technologies for regenerative medicine. This may include the development of advanced tissue and organ preservation technologies and processes for cell banking, such as specimen harvest, cell retrieval, selection and expansion, tissue typing, storage, and distribution. Promising paths include the development of new and more stable dimethyl sulfoxide-free stabilizer formulation compositions, lyophilization formulations, and characterization of the effects on the product (cellular function) both pre- and post-thaw identification of reagents that serve to preserve cellular function upon freeze drying. Additionally, novel drying technologies that optimize formulations for reconstitution are needed. Specialized packaging for cells, tissues, and organs is needed, as well as training to properly employ their technologies, as well as appropriate infrastructure at point-of-care facilities.

Solutions may utilize short-term cryostorage coupled with new techniques for distribution. Such hybrid storage would enable initial cryogenic storage followed by noncryogenic distribution for high-quality products. Customized designs for closed preservation systems coupled with sensors may also be employed to monitor quality and enable longer preservation time.

GLOBAL LANDSCAPE FOR REGENERATIVE MEDICINE MANUFACTURING

The global landscape for regenerative medicine manufacturing is exciting and filled with tremendous potential. Next we will highlight some of the technical societies that are forming in this area, as well as the numerous manufacturing-focused initiatives that are being launched around the world. Table 78.5 lists the initiatives, focus areas, and websites where you can obtain additional information.

Technical Societies

There is a new technical society recently formed called the Regenerative Medicine Manufacturing Society (RMMS). RMMS is a new organization that has a vision of enabling the adoption of manufacturing platform technologies into standards, regulatory pathways, and commercial products by assembling a diverse network of stakeholders. These stakeholders fall into these major categories: (1) industry, (2) academia (basic and clinical research), (3) regulatory agencies, (4) nonprofit organizations, and (5) investment and funding agencies. RMMS has a strategic goal to bring these stakeholders together to achieve a number of regenerative medicine manufacturing aims, including the following: (1) enabling the development of scale-up tools and reagents; (2) enabling the development of standards; and (3) seamless integration of platform technologies with regulatory pathways. Additional information on this new technical society can be found at http://regenmedmanufacturing.org/. The first RMMS conference

TABLE 78.5 Global Landscape in Regenerative Medicine

Initiative	Focus Areas	Website
Medical Technology Enterprise Consortium	Regenerative medicine technologies and manufacturing	https://mtec-sc.org/
National Institute for Innovation in Manufacturing Biopharmaceuticals	Biopharmaceutical manufacturing	http://www.niimbl.us/
Biofab USA	Biofabrication	https://www.armiusa.org/
Centre for Commercialization of Regenerative Medicine	Cell and gene therapies Regenerative medicine technologies	https://ccrm.ca/
Catapult	Cell and gene therapy	https://ct.catapult.org.uk/
Japan—regulatory	New regulations to accelerate regenerative medicine therapeutics	
Regenerative Medicine Manufacturing Society	Regenerative medicine manufacturing	http://rmmanufacturingsociety.org/about/

was held in Miami, Florida, in January 2018 in concert with the World Stem Cell Summit and brought together leaders from industry, academia, and government.

Domestic Efforts

Efforts currently under way in the United States to advance regenerative medicine manufacturing are growing every day.

21st Century Cure's Act, also known as the Cures Act, was signed into law on December 13, 2016, with the intention of assisting the acceleration of medical product development and bringing new advances to patients in a faster, more streamlined manner. The Cures Act seeks to incorporate the perspectives of patients into the development of drugs, biological products, and devices in the US FDA's regulatory process. The Cures Act has the intention of speeding up the development and review of novel medical products (including medical countermeasures) and gives new authority to assist the FDA in building and retaining scientific, technical, and professional experts by establishing new product development programs such as (1) RMAT, a new expedited option for eligible biologics, and (2) the Breakthrough Devices Program to accelerate the review of innovative medical devices. The Cures Act also charges the FDA with improving the regulation of combination products by creating one or more intercenter institutes to coordinate activities in major disease areas between the drug, biologics, and device centers. In total, the Cures Act provides $500 million over 9 years to assist the FDA in implementing this law to accelerate the discovery, development, and delivery of 21st century cures. Currently, the FDA has submitted a work plan to Congress that lays out a budget for advancing seven strategic areas of activity: (1) patient-focused drug development, (2) advancing new drug therapies, (3) modern trial design and evidence development, (4) patient access to therapies and information, (5) antimicrobial innovation and stewardship, (6) medical device innovations, and (7) improving scientific expertise and outreach at the FDA.

The National Network for Manufacturing Innovation (e.g., Manufacturing USA) has funded two institutes dedicated to advance regenerative medicine manufacturing through public/private partnerships. These institutes join 12 others, each focusing on a technology or industry group by bringing together industry, academia, and government to increase competitiveness through applied research, outreach, and workforce development.

The National Institute for Innovation in Manufacturing Biopharmaceuticals (NIIMBL) has a mission "to accelerate biopharmaceutical manufacturing innovation, support the development of standards that enable more efficient and rapid manufacturing capabilities, and educate and train a world-leading biopharmaceutical manufacturing workforce, fundamentally advancing U.S. competitiveness in this industry" [17]. NIIMBL will initially seek to understand biomarkers for potency, develop sensors for these biomarkers, and use this knowledge and developments for cost-effective scale-up for mesenchymal stem cells, T-cells, and induced pluripotent stem cells.

The Advanced Regenerative Medicine Institute "will make practical the large-scale manufacturing of engineered tissues and tissue-related technologies, to benefit existing industries and grow new ones" [18]. The institute seeks to develop innovations across five thrust areas: (1) cell selection, culture, and scale-up; (2) biomaterial selection and scale-up; (3) tissue process automation and monitoring; (4) tissue maturing technologies; and (5) tissue preservation and transport.

The Medical Technology Enterprise Consortium (MTEC) is a nonprofit biomedical technology consortium that collaborates with multiple government agencies under a 10-year renewable Other Transaction Agreement with the US Army Medical Research and Material Command. MTEC fosters integrated research partnerships to provide solutions to military, veterans, and the civilian population. Consortium thrust areas include: (1) prevention, diagnosis, and treatment of infectious diseases, (2) care of combat casualties, (3) clinical and rehabilitative medicine, (4) military operational medicine, (5) medical simulation and information sciences, and (6) advanced medical technologies. Significant emphasis is placed on aspects of regenerative medicine that will facilitate manufacturing scale-up.

International Efforts

There are also many regenerative medicine international efforts and three specific efforts that we will focus on here include: (1) the Centre for Commercialization of Regenerative Medicine (CCRM), (2) Catapult, and (3) Japan.

CCRM is a nonprofit, public/private consortium whose mission is to generate sustainable health and economic benefits through global collaboration in cell and gene therapy, as well as regenerative medicine. CCRM has recognized there is an unmet need in advancing cell, gene, and regenerative medicine-based treatments to the clinic. This

Canadian-based effort uses stem cells, bioengineering, and biomaterials combined with their consortium and infrastructure to coordinate product development and commercialization.

The Cell and Gene Therapy Catapult is a network of centers in the United Kingdom designed for innovation in specific strategic areas to promote future economic growth. Each Catapult center specializes in a different area of technology, including areas such as cell and gene therapy, high-value manufacturing, transport systems, medicines discovery, and others. This Catapult network provides access to technical capabilities, equipment, and additional resources to develop new products and services on a commercial scale.

Japan is advancing regenerative medicine by enabling early commercialization and early reimbursement. This effort is in response to half the population of Japan, which is over age 50, and regenerative medicine-based therapies have been limited in the past because of the difficulty in progressing through Japan's Pharmaceuticals and Medical Devices Agency. Prime Minister Shinzō Abe has launched an economic revitalization plan that includes ¥110 billion ($1 billion) in funding for stem cell research. In addition, two new regenerative medicine laws were enacted. Law No. 84/2013 provides an amendment for the Pharmaceutical Affairs Act, renamed the Pharmaceutical and Medical Device Act, and provides conditional marketing authorization. This new regulation provides revenue for regenerative medicine products as soon as this conditional approval is granted, which could be achieved after a small patient number phase 1 safety trial. Law 85/2013 is part of the Safety of Regenerative Medicine Act, and is for clinical and physician-led research. This Act focuses on the cells and allows them to be processed outside hospitals at accredited cell-processing centers to enable manufacturing processes that are more robust with the intention of promoting a safer product through a tier-based risk-dependent analysis.

SUMMARY AND CONCLUSIONS

This chapter on regenerative medicine manufacturing presented a comprehensive overview of current challenges and opportunities that currently exist in this space. We have also provided a perspective on envisioned regenerative medicine manufacturing systems of the future with focus on specific attributes that we believe will be critical for widespread adoption of regenerative medicine-based technologies and products. Moreover, we briefly described the global landscape for regenerative medicine manufacturing and highlighted some of the technical societies and efforts currently under way.

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PRINCIPLES OF REGENERATIVE MEDICINE
THIRD EDITION

Edited by Anthony Atala, Robert Lanza, Antonios G. Mikos, and Robert M. Nerem

Research and clinical applications in the expansive field of regenerative medicine incorporate therapies associated with any disease that results from malfunctioning, damaged, or failing tissues. From cell treatments to engineering damaged blood vessels, corneas, and organs for transplantation into human patients, regenerative medicine is changing the face of health care and expanding the possibilities for the translation of therapies. Now in its third edition, *Principles of Regenerative Medicine* remains the most comprehensive book in the field. This new edition in the expansive field of regenerative medicine has been fully updated and details the technologies and advances applied in recent years to strategies for healing and generating tissues. Contributions from a stellar cast of researchers, clinicians, and industry and government representatives cover the biological, molecular, cellular, and biomaterial bases of regenerative medicine, highlighting stem cells and wound healing, cell and tissue development, and a variety of enabling technologies such as three-dimensional printing. Advances in cell and tissue therapies, including the restoration of tissues and organs damaged by disease such as diabetes, heart disease, liver disease, and renal failure, are incorporated to provide a view to the future and a framework for additional studies. Topics such as regulatory and commercial parameters, including biomanufacturing, are also covered, providing an in-depth look at the field of regenerative medicine from the basic sciences to clinical translation and commercialization. This textbook would be beneficial to students, researchers, clinicians, industry and government representatives, and members of the public at large who are interested in the topic.

- Comprehensively covers the interdisciplinary field of regenerative medicine with contributions from leaders in stem cell biology, tissue engineering, cell and developmental biology, biomaterials sciences, nanotechnology, physics, chemistry, gene editing, bioengineering, and surgery
- Includes a large number of new chapters devoted to the most recent advances in the field of regenerative medicine by the scientists who made the breakthroughs
- Edited by a world-renowned team to present a complete story of the development, progress, and promise of regenerative medicine

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