Stem Cell Biology and Regenerative Medicine

Tarun Pandey *Editor*

Imaging in Stem Cell Transplant and Cell-based Therapy

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Preface

Stem cell therapy has made rapid strides in recent years and has generated considerable interest among scientific communities, clinicians and general public. A lot of attention emanates from the great promise offered by this technique, most notably with regard to the application of stem cell therapy for diseases that are currently difficult to treat or incurable. Hence, study of stem cells and cell-based therapies, that was traditionally viewed as a core research area and thought to be of interest mainly to the researchers and scientists, is fast getting into the paradigm of clinical care.

For diagnostic and interventional radiologists, it is particularly advantageous to be actively involved in the bench to bedside development of these therapies. While diagnostic radiologists can become experts in imaging, tracking, and monitoring of stem cells and in the assessment of engraftment efficiency, the interventional radiologist can play an important role in targeted stem cell delivery by means of different routes (percutaneous, selective intravenous, or intra-arterial).

The prevailing literature in stem cell therapy predominantly targets the core researchers. Also, the subject matter is complex, has abundant technical jargon that is somewhat difficult to comprehend for an average clinician. This book will present a simplified review of stem cell and cell-based therapies with a focus on imaging.

The current volume will provide a review of imaging techniques and applications in stem cell transplantation and other cell-based therapies. The basis of different molecular imaging techniques will be explained in detail. Applications of stem cell transplant in different organ systems will be discussed, with reference to imaging wherever feasible. The reader can expect to get comprehensive information on the role of clinical and molecular imaging in stem cell therapy from this book. This book will provide opportunities to learn the current gamut of stem cell applications in adults and pediatric populations, understand the scope of molecular imaging in stem cell and cell-based therapies, understand role of clinical imaging in stem cell therapy and gain knowledge in several state of the art applications in this field like use of nanotechnology in stem cell applications, stem cell use in cardiology, cancer and angiogenesis. This information will be presented in a simplified form that will generate reader interest in this technology.

The work is primarily targeted towards radiologists and physicians involved in molecular imaging who are interested in developing a basic understanding of stem cell imaging and applications of stem cells and cell-based therapies. However, it will also be of interest to clinical scientists and researchers alike. A variety of physicians can benefit from this volume including, but not limited to, radiologists, cardiologists, hematologists, interventionists and transplant physicians. PhD researchers involved in stem cell labeling, tracking and imaging, cancer therapy, angiogenesis and cardiac regeneration will find this issue highly useful.

The book is organized in two main sections. Section 1 provides overview of stem cell indications and techniques in adult and pediatric population with review of molecular imaging techniques and shall discuss role of newer applications like use of nanotechnology in stem cell transplant. Section 2 is devoted to review common applications in stem cell and cell-based therapies. Overall, the objective is to provide a unique resource, focusing on imaging in stem cell and cell-based therapies with review of stem cell therapy applications and to provide simplified explanation of technical concepts and terminology. The text is written from a clinician's perspective that will help the average physician to keep abreast with stem cell research and encourage him/her to adopt this technology.

While each section will have a hierarchical organization of reviews encompassing basic to advanced topics, each chapter will be self-sufficient enough so that it can be reviewed independently of one another.

The text is compiled by a variety of authors who bring in years of experience in stem cell research and clinical imaging. The cross-disciplinary expertise of the contributors will ensure that the book will present a balanced perspective. The authors of this book are national leaders in their respective areas and well known internationally through their work and citations. The contributors span the entire length and breadth of United States with several overseas contributors as well. Also, they represent premier organizations and universities in the United States and abroad, well known for quality academics and research work in stem cells.

I hope that this work will accomplish its objectives. Like any similar book project this would not have reached fruition but for the support from the contributing authors. I wish to thank all authors for their hard work in compiling the chapters and sharing their expertise with the world. I am extremely grateful to the Series Editor of *Stem Cell Biology and Regenerative Medicine*, Dr. Kursad Turksen for giving me this opportunity to edit this series. Last but not the least, I am ever grateful to my parents, lovely wife and sons for their continued support, and to my students who have kept me motivated all these years!

Little Rock, AR, USA Tarun Pandey

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About the Editor

Tarun Pandey, M.D., F.R.C.R. is an Associate Professor of Radiology at the University of Arkansas for Medical Sciences. His clinical work is focused on magnetic resonance imaging. Throughout his career, Dr. Pandey has had varied interests ranging from musculoskeletal MRI, body imaging and cardiac MRI. His current area of research includes molecular imaging and its applications in cardiac and cancer imaging, especially in diagnosis and treatment of multiple myeloma. He is the fellowship director of the MSK/Body MRI fellowship at the University of Arkansas for Medical Sciences and enjoys teaching his fellows and residents. He has authored more than fifty peer-reviewed articles, reviews for numerous journals, and has served several educational committees at national and international radiology societies. The focus of his teaching is to simplify complex educational content that will stimulate reader interest. With this book on stem cell imaging, Dr. Pandey brings this subject to the imaging mainstream.

Part I
Imaging in Stem Cell Transplant

Chapter 1
Current Indications and Overview of Molecular Imaging Techniques in Stem Cell Transplantation

Tarun Pandey

1.1 Introduction

Stem cell transplant has been a focus of clinical research for a long time given its immense promise to treat several difficult to treat and incurable diseases like hematological malignancies, diabetes mellius and neurodegenerative disorders like Parkinson's disease. Recently there has been a renewed interest in this technique and expansion in applications due to advancement in various stem cell technologies. Traditionally viewed as a core research area; the study of stem cells and cell-based therapies is no longer limited to basic researchers and scientists and is fast getting into the paradigm of clinical care.

Stem cell imaging is a fast growing niche area in this field. It encompasses a wide spectrum including molecular imaging, diagnostic and interventional radiology. Molecular imaging offers diverse imaging applications including imaging, tracking, and monitoring of stem cells and in the assessment of engraftment efficiency. Rapid strides in imaging techniques related to stem cell harvesting, labeling, tracking, engraftment and monitoring of treatment response has allowed imaging to gain a central role in stem cell research and development. Minimally invasive Interventional radiology techniques have also been developed that help in engraftment of the administered stem cells. The interventional radiologist can also be highly valuable in targeted stem cell delivery by means of different routes (percutaneous, selective intravenous, or intra-arterial).

In spite of these advances, stem cell transplant has many challenges. Success rate with this technique has not been universal and many complications have also been seen with this form of therapy. Hence diagnostic radiologists play an important role

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in imaging of transplant diseases, especially in the diagnosis and management of complications associated with stem cell transplantation [1].

This chapter shall briefly review the history of stem cells and their current status and applications. We shall also discuss the role of radiology and interventional techniques in administration and monitoring of transplanted stem cells. Recent advances in stem cell techniques and imaging of the stem cell transplant in various areas shall be discussed here and in the subsequent chapters.

1.1.1 Stem Cell Types: Definitions and Classification

Stem cells are undifferentiated biological cells that can differentiate into specialized cells and can undergo mitosis to produce more stem cells. Regardless of their source, all stem cells have three general properties: they are capable of dividing and renewing themselves for long periods (long term self-renewal); they are unspecialized i.e., they lack tissue specific structure and function (plasticity); and they can give rise to specialized cell types (differentiation) [2, 3].

Stem cells are distinguished from one another on the basis of their plasticity. Not all stem cells have the same degree of plasticity, or developmental versatility. Some stem cells are more committed to becoming any particular type of cell than others. The categories into which the various stem cells fall include: the totipotent stem cell,

Table 1.1 Summary of different stem cell imaging strategies with emphasis on the underlying physiological process and role of different modality in each strategy. A comparative estimate of the spatial resolution and stem cell detection sensitivity in the direct and indirect labeling of the stem cells is also provided. (+ least resolution/sensitivity, ++++ maximum resolution/sensitivity). Notice spatial resolution and stem cell detection sensitivity is not applicable to the direct transfer of stem cells given on-site delivery of stem cells using interventional techniques. [MRI = Magnetic resonance imaging; PET = Positron emission tomography; SPECT = Single photon emission computed tomography). [Adapted from Rodriguez-Porcel M, Wu JC, Gambhir SS. Molecular imaging of stem cells. In Stembook. http://www.stembook.org/node/603. Last accessed 03/15/2014.]

Stem cell imaging strategy	Underlying physiological process	Imaging modality	Spatial resolution	Stem cell detection sensitivity
Direct stem cell labeling	1. Early stem cell detection 2. Stem cell homing	1. Fluorescence 2. MRI 3. SPECT 4. PET	++ ++++ +++ +++	+++ +++ +++ +++
Indirect stem cell labeling (reporter gene imaging)	1. Long term monitoring of cell viability 2. Study stem cell biology	1. Fluorescence 2. Bioluminescence 3. SPECT 4. PET 5. MRI	++ ++ +++ +++ ++++	+++ ++++ +++ +++ Not known
Direct transfer of stem cells	Direct delivery of stem cells to target area	1. MR Fluoroscopy 2. Ultrasound	NA	NA

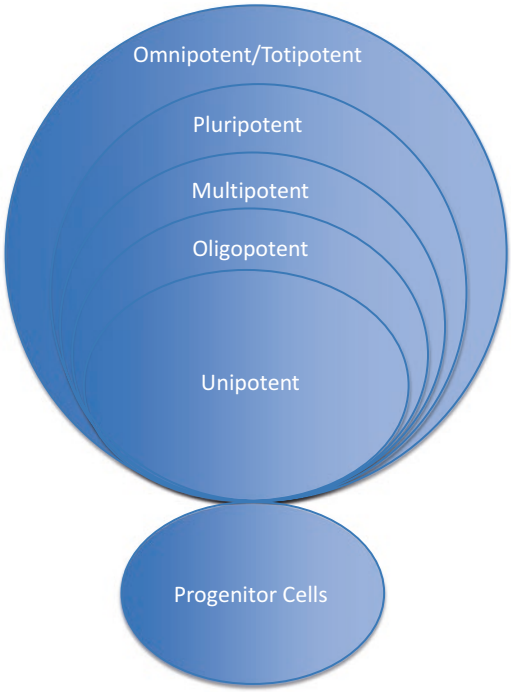

Fig. 1.1 Simplified representation of the stem cells and their lineages in the body

pluripotent stem cell, multipotent stem cell, and the adult stem cell (a certain type of multipotent stem cell). These properties of stem cells are described in Table 1.1.

Embryonic stem cells are examples of totipotent stem cells, allowing them to give rise to any mature cell type. This latter property implies that an entire organism can be constructed from these embryonic stem cells. Adult stem cells are examples of Pluripotent stem cells, very similar to totipotent stem cells in that they can give rise to all tissue types. But unlike totipotent stem cells they cannot give rise to an entire organism. Multipotent cells can differentiate into a number of cell types, however, these are closely related family of cells. Oligopotent cells are further limited to differentiate into only a few cell types (e.g., lymphoid or myeloid cells), whereas unipotent cells can only produce one cell line. It is important to note that the property of self-renewal in stem cells is unlimited. This differentiates them from other non-stem cells like progenitor cells that have a limited capacity of self-renewal. Based on their source stem cells can be classified into two broad categories. Embryonic stem cells, found in the inner mass of the blastocyst and adult stem cells, found in various mammalian tissues. Figure 1.1 presents a simplified representation of the stem cells and their lineages in the body.

1.1.2 History of Stem Cells

The term "stem cell" was proposed for scientific use by the Russian histologist Alexander Maximow in 1908. He postulated existence of hematopoietic stem cells. In 1959, E. Donnall Thomas performed successful syngeneic bone marrow transplant in two patients with acute lymphoblastic leukemia. He shared the Nobel Prize in physiology in medicine with Joseph Murray, a surgeon instrumental in the development of kidney transplantation [4]. In 1968 bone marrow transplant between two siblings was used to successfully treat severe combined immunodeficiency. For most of the latter half of the previous century stem cell transplant techniques focused on hematopoietic stem cells and bone marrow transplant. It was not until 1998 when Thomson and co-workers derived the first human embryonic stem cell line [5]. Since then several rapid strides have been made in this arena. Several new sources of stem cells have been shown like primary teeth [6] , cord blood, amniotic fluid [7]. In 2007 Mario Capecchi, Martin Evans, and Oliver Smithies were awarded the 2007 Nobel Prize for producing genetically engineered mice (known as knockout mice) using embryonic stem cell gene targeting.

1.1.3 Clinical Applications of Stem Cells

Stem cells can be used for a variety of applications. Some of the uses are summarized below:

1.1.3.1 Understanding Genetic and Molecular Controls of Cell Division and Differentiation

The control of cell division and differentiation is dependent on an orderly control of the genes. Understanding how undifferentiated stem cells become differentiated to form tissues and organs is central to the study of abnormal cell division in conditions like cancer and birth defects. The information gained from the study of the genetic and molecular mechanisms underlying cell division and differentiation may help better understand the molecular basis of these diseases and can suggest novel therapeutic approach.

1.1.3.2 Drug Testing

The safety and efficacy of new drugs can be tested on differentiated cells generated from human pluripotent cell lines. Similarly, cancer cell lines are used to screen potential anti-tumor drugs. Pluripotent cell lines can generate a number of differentiated cell types, allowing a variety of cell substrate for drug testing.

1.1.3.3 Cell-Based Therapy

Autologous or allogeneic hematopoietic stem cell transplantation is now a routine procedure and has been successfully used for treatment of variety of hematological conditions such as lymphoma, multiple myeloma, leukemia, anemias, solid tumors like neuroblastoma and germ cell tumors. Also selected autoimmune conditions like systemic lupus erythematosus have also been treated using stem cells. According to estimates more than 50,000 autologous and allogeneic transplantation procedures are performed every year worldwide [8].

The current interest in stem cell transplant has evolved and the research focus shifted to use the general capacity of stem cells to repair or restore damaged tissue and recover lost function. The prospect of using undifferentiated stem cells to maintain, recover and improve organ function has opened a new era of research especially in diseases when there is no cure, alterative treatment or where organ transplant is not an option. Approximately 128 million people suffer from chronic, degenerative, and acute diseases, and stem cell therapies hold great promise in the treatment of many of these diseases. Advances in stem cell biology have allowed expanded the use of stem cells to treat non-malignant diseases. These applications include treatment of autoimmune diseases, restoring or normalizing hematopoietic function, and treating inborn errors of metabolism. The goal of immunoablative therapy followed by HSCT is aimed at resetting the patient's immune system and allow outgrowth of a nonautogressive immune system from reinfused hematopoietic stem cells allowing the immune system to shift from a highly pro-inflammatory disease environment to a less inflammatory one [9, 10].

Both autologous and allogeneic stem cells can been used to treat various immune mediated diseases including multiple sclerosis (MS), systemic sclerosis (SSc), systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) [11].

Stem cell derived cells; tissues and possibly organs may become a renewable source of replacement cells and tissues to treat diseases including cancer, diabetes, macular degeneration, spinal cord injury, stroke, burns, heart disease, osteoarthritis, and rheumatoid arthritis. However, ethical concerns have also been raised in the use of stem cells, especially use of embryonic stem cells, for purposes of research and disease treatment. The idea of sacrificing human embryo has prevented clinical trials using embryonic stem cells in countries like the United States. The treatment efficacy of stem cells in tissue repair is also questionable fuelling such concerns. Overall, there has been scientific disagreement about the ability of hematopoietic stem cells to differentiate into cells that could impact tissue angiogenesis and regeneration. Clinical trials with systemic and direct injection of stem cells to achieve repair have shown mixed results. The PROTECT-CAD trial showed the most promising results, in which bone marrow mononuclear cells were injected directly into ischemic myocardium in patients with refractory myocardial ischemia [12].

However, on-going research in this area has now made it possible to produce a stem cell from almost any other human cell instead of using embryos as needed previously [13]. This has alleviated some of the ethical concerns and has created more opportunities in use of stem cells for tissue repair and regeneration.

Recently, stem cells have been successfully used in treatment of non-hematological conditions in humans with successful cartilage regeneration in

human knee using autologous adult mesenchymal stem cells [14]. Subsequently, promising results in human clinical trial in treating type 1 diabetes has been shown using Cord Blood-Derived Multipotent Stem Cells (CB-SCs) [15].

A detailed discussion on some of these applications can be found in subsequent chapters of this book.

1.2 Imaging in Stem Cell Transplant

The role of imaging can be broadly categorized in two categories, encompassing a wide spectrum. At a molecular level, stem cell imaging includes processes like labeling, homing and longitudinal monitoring of the stem cells. On the other hand, at a macro or organ/system level, imaging has a crucial role in evaluation of stem cell transplant related complications. An imaging specialist is ideally positioned to become an integral part of stem cell imaging in various capacities. As a molecular scientist, he or she can label, track stem cells, monitor and evaluate the engraftment efficiency. Also, as interventionists, radiologists can play key role in targeted delivery of stem cells by different routes. As clinical radiologist he or she is not infrequently called upon to evaluate and discuss various stem cell transplant related complications.

Traditionally, imaging in stem cell transplant was focused on monitoring of complications like host versus graft disease as most stem cell transplants were performed for hematological indications. The advent of targeted stem cell delivery in non-hematological stem cell applications has opened new applications of imaging and shifted focus toward monitoring of the graft and evaluating graft engraftment and site-specific complications. In some cases interventional radiology expertise is needed prior to stem cell transplant to create engraftment territory and increase engraftment bed fertility with controlled intentional tissue destruction, like thermal ablation [2]. The rest of this chapter will present a systematic review of imaging in stem cell transplant and will address role of conventional and molecular imaging in both targeted and systemic stem cell therapy. Recent advances such as role of nanotechnology will be addressed in a dedicated chapter.

1.3 Use of Imaging Techniques in Stem Cell Transplant

The rapid strides in molecular imaging in recent years have allowed successful non-invasive imaging of the transplanted cells. The role of these imaging techniques to study the viability and biology of the transplanted cells is critical to the study of different regenerative therapies.

Imaging not only allows monitoring of stem cell transfection, uptake and homing to target area but is also capable of longitudinal evaluation of the stem cells in a living subject and shows interaction of the cells at the level of the microenviron-

ment. Various single and multimodality imaging strategies have been adopted that form the cornerstone of translational research with bench to bedside applications in clinical care.

Any modality that needs to be used for stem cell imaging should fulfill several criteria. It should be capable of real rime visualization of stem cell delivery. Once stem cells are implanted it should be able to locate them at the target site. It should also allow quantification of viable stem cells and estimate long-term survival. Finally the modality should be able to study stem cell biology, like interaction of stem cells among one another and with their microenvironment. Moreover, the modality itself should not alter the stem cells in any way. The method of stem cell labeling employed by a particular modality must be biocompatible with minimal toxicity when released by the stem cell. If an intermediary agent is required for this process then that agent must not interfere with normal regulatory or differentiation pathways of the cells. Moreover, the imaging technique must be sensitive enough to the label such that it can still be detected, at least following initial stem cell division and replication.

Three different strategies are used for stem cell labeling. The first involves direct non-specific labeling of the stem cells. The second method involves an indirect, receptor mediated specific cell labeling and the third technique employs a reporter gene probe labeling [16]. Typically a labeling agent is introduced into the stem cells prior to transplantation. Following transplantation of the cells non-invasive imaging is performed. The modalities that are most useful in direct stem cell labeling include Optical imaging, MRI, PET, SPECT and ultrasound. The appropriate labeling strategy for each modality is discussed below. A summary of the various stem cell imaging strategies is shown in Table 1.1**.**

1. **Magnetic Resonance Imaging (MRI):** Magnetic resonance imaging is the most extensively studied technique to directly label and image stem cells non-invasively [16]. MR imaging can utilize both T1 and T2* properties to label stem cells. T1 agents employ use of paramagnetic metal chelates like gadolinium to directly label stem cells. Contrary to expectations, a T1 agent does not produce a hyper intense signal when internalized inside the stem cell. This is due to reduced water exchange across the cell membrane limiting the “conventional” dipole-dipole interaction. In this situation the outer sphere magnetic susceptibility predominate resulting in a hypo-intense signal [17]. However, the pharmacokinetics of the T1 W agents is not predictable. For example the de-chelated form of gadolinium is highly toxic. On the other hand, the T2* properties of the super paramagnetic iron oxide (SPIO) contrast agents is robust and extensively studied in labeling of the stem cells.

   (a) MRI Labeling techniques:

Stem cells are non-phagocytic and must be induced to incorporate the SPIOs. This can be accomplished using any of the following approaches. Magnetofection is a technique where the negatively charged SPIO is mixed with a positively charged transfection agent. The electrostatic interactions create a SPIO-Transfection agent complex that is incubated with stem cells for 24 h allowing migration of the complex into the stem cells through invagination or pinocytosis. The second technique involves use of low voltage electromagnetic pulses to transfect stem cells with proteins or DNA. It is called magneto-electroporation (MEP) and is much faster than magnetofection, allowing stem cell labeling in a minutes and does not need a transfection agent. Both techniques allow stable uptake of SPIO's in stem cells. Recently, a new instant magnetic cell labeling technique, called magnetosonoporation (MSP) has been described that uses ultrasound waves instead of electric pulses to temporarily permeabilize cells (sonoporation), enabling intracellular uptake of exogenous compounds [18]. MSP can instantly label a large amount of stem cells without use of a transfection agent.

Direct labeling techniques have a drawback of not mirroring the cell physiology. SPIOs may remain in stem cells even after cell death creating false impression of cell viability. Some SPIOs may be engulfed by macrophages instead of stem cells confounding the data. Also, dilutional effects of cell division may make labeled cells undetectable after several generations. In spite of the drawbacks and limitations of direct labeling techniques, SPIO labeled mesenchymal stem cells have been shown to generate good results up to 8 weeks in animal models of myocardial infarction [19, 20].

Indirect labeling is typically accomplished by binding an MR contrast agent to a stem cell specific receptor. Receptor based imaging is more sensitive compared to internalization of contrast in the direct methods and also more specific since the receptor is unique to the target cell. While indirect labeling techniques hold promise but currently no receptor based cell labeling have been successfully applied using MRI.

Reporter gene cell labeling is another method of labeling the stem cells. In this approach the stem cell is transfected with a gene that expresses an enzyme, protein or a receptor that can be detected using MRI [21, 22]. The potential advantages of this technique are many including, indefinite tracking of cells due to lack of dilutional effects from cell division, dead cells don't confound results as only viable cells express the signal protein or receptor. Also, the reporter gene in a stem cell can be programmed to signal only if a particular condition is met, like when the stem cell undergoes differentiation to the intended mature cell (stem cell maturing into chondrocyte or cardiac cell etc.). Most MRI based reporter genes are based on production of intracellular metalloproteins, mostly transferrin, ferritin and tyrosinase. Increasing the transferrin expression using a reporter gene will lead to accumulation of intracellular iron that can be detected using MRI by studying changes in the T2* signal (Fig. 1.2). Similarly, tyrosinase reporter gene produces melanin that binds iron inside the cells that leads to increased relaxivity. Using iron

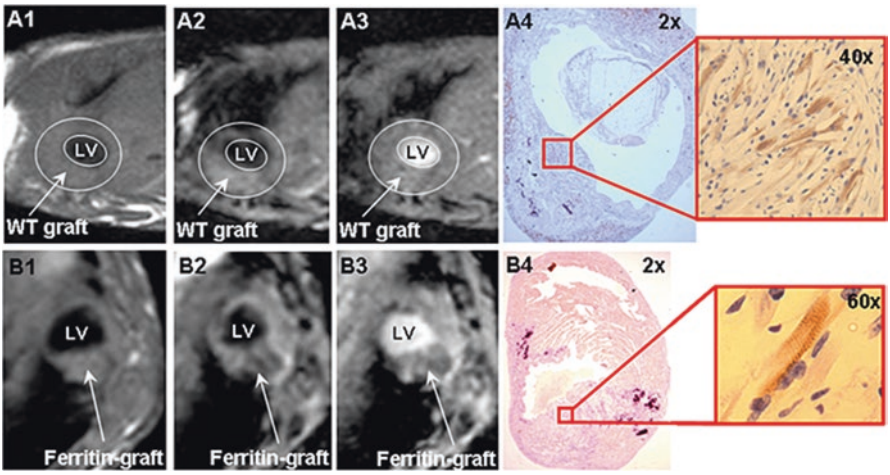

Fig. 1.2 MRI detection of ferritin-tagged graft in the infarcted mouse heart. (*A1–A4*) Unlabeled wild-type C2C12 graft. (*B1–B4*) Transgenic C2C12 graft overexpressing ferritin. (*A1*, *B1*) Black-blood proton-density-weighted turbo spin echo (PD TSE BB) sequence. (*A2*, *B2*) Black-blood improved motion-sensitized-driven equilibrium (iMSDE) T2* sequence. (*A3*, *B3*) Bright-blood gradient echo (GRE) T2* sequence. (*A4*, *B4*) Embryonic myosin immunostaining for graft detection. *LV* left ventricle. (Courtesy, Naumova A et al. Quantification of MRI signal of transgenic grafts overexpressing ferritin in murine myocardial infarcts. NMR Biomed. 2012 Oct;25(10):1187–95)

based metalloproteins for MR imaging has its own drawbacks, especially related to toxicity from iron accumulation [22]. There are also concerns regarding signal nonlinearity. Dilutional signal loss following cell division takes time to build up to detectable levels making correlation between detected signal and viability of transplanted cells difficult. The R2 relaxivity of iron is dependent on concentration. With very high iron accumulation T2 relaxation plateaus and precludes accurate quantification of the signal. Following cell death the reporter gene does not function but the iron already accumulated iron inside the cell generates signal, similar to SPIOs. Hence in such situations the signal is neither representative nor linear of cell viability [23]

2. **Optical Imaging***:* Optical imaging comes in two flavors, bioluminescence and fluorescent techniques. Bioluminescence as the name suggests is a technique where the source of light is within the organism. In this technique, a light source is incorporated in the organism using DNA encoding of the luminescent protein. The protein is incorporated in the stem cells, typically using a viral vector creating a reporter gene. There are three common organisms that provide the light source. Firefly luciferase (D-luciferin) from north American firefly, Luciferase from Sea Pansy like Renilla luciferase (coelenterazine) and Bacterial Luciferase from bacteria like photorhabdus luminescent or vibrio fischeri. The light is produced by the substrate independently or in conjunction with cofactors. For example oxidation of the substrate D-luciferin by the firefly luciferase (FLuc) enzyme

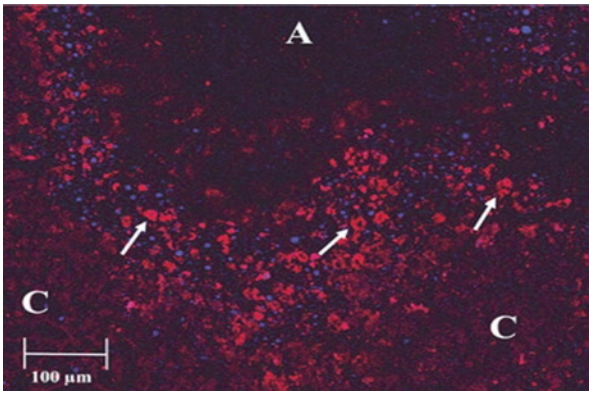

Fig. 1.3 Antibody-stained fluorescent images after RF ablation (magnification 20, zoom ×0.7) show stem cell uptake at coagulation margin with fluorescent stem cells (*arrows*), coagulation area (*A*) and the more peripheral hepatic parenchyma not subjected to substantial changes from RF ablation heating (*C*). Focal *blue* areas of fluorescence represent 4′,6-diamidino-2-phenylindole stain of nuclei as anatomic markers and are unrelated to stem cell labeling. (Courtesy, Nikolic B et al. The effect of hepatic radiofrequency ablation on stem cell trafficking in the rat model. J Vasc Interv Radiol. 2009 May;20(5):640–7)

in combination with oxygen, magnesium and ATP results in a reddish light (500–700 nm).

On the other hand, fluorescent techniques depend on incorporating an inorganic substrate into the cells that have fluorescence properties that is they emit light when excited by external light or wavelengths. These substrates can be organic, like green fluorescence protein or small molecule polymethines or inorganic semiconductor nanocrystals (quantum dots) [24].

Optical bioluminescence imaging has been successfully used in molecular imaging of stem cells [2, 25] (Fig. 1.3). However there are certain limitations of this technique. Firstly, only visible light is generated by the luciferase (400–700 nm wavelength). Also, the light is produced in small quantities requiring an ultra-sensitive charge coupled device (CCD) camera to generate images. But more importantly, optical imaging has limited tissue penetration (around 2 mm) restricting the use of the technique to superficial tissues and small animals like mice and rat [26]. The technique is also a planar imaging with no limited depth perception and resolution (4–10 cm). There is also signal loss due to subsequent cell divisions and possible stem cell uptake by macrophages after stem cell death [27].

3. **Radionuclide Imaging:** Use of radionuclides for labeling and imaging of stem cells also employs several strategies. Direct labeling techniques similar to SPIOs can be used where the radiotracer is introduced into the stem cells prior to transplantation. The radionuclides emit gamma radiation that is imaged using a gamma camera and/or SPECT. Common radionuclides are Tc99m (Half life 6 h) and In111 (Half life 2.8 days). Some radionuclides (like F-18-Fluoro-

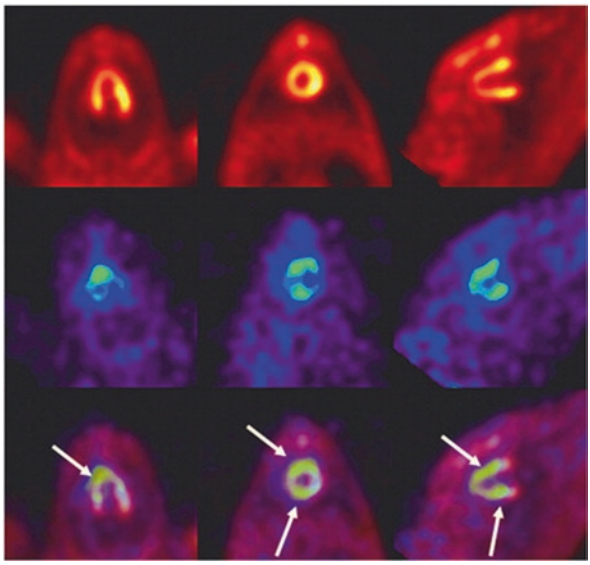

Fig. 1.4 Comparison of 18F–FDG PET (*upper row*) and 18F-FDG-labeled MSCs (*middle row*) in a rabbit myocardial infarction model. Three days after ligation at the left anterior descending artery near the apical region, 1.110718F-FDG-labeled MSCs were injected directly into the left ventricle. The PET scan was performed 3.5 h after injection. In the fusion images (*lower row*), 18F-FDG-labeled MSCs were found to be distributed into the peri-infarcted zone of the myocardium (*arrow*). (Courtesy, Wu C et al. In vivo cell tracking via ^{18}F–fluorodeoxyglucose labeling: a review of the preclinical and clinical applications in cell-based diagnosis and therapy. Clin Imaging. 2013 Jan-Feb;37(1):28–36)

Deoxyglucose) are positron emitters and decay by emitting high-energy gamma rays that need a PET scanner to generate images. The extent of monitoring of the stem cells using this technique is time limited and depends on the half-life of the radionuclide. In general the time available for imaging is the lesser of the physical and biological half-life of the radionuclide. Radionuclide imaging has been used successfully to label and image stem cells in cardiac applications and imaging of myocardial infarction [28, 29]. The major advantage of using SPECT or PET imaging is the their ability to detect very low quantities of radiotracer. As low as nano and femto-molar concentrations can be detected respectively by these modalities. However these modalities are limited by relatively low spatial resolution when compared to MRI. Combining PET and SPECT with CT has helped remove overlapping anatomy but spatial resolution remains inherently poor.

Reporter gene imaging using SPECT and PET has also been successfully employed in the past [30, 31]. A reporter gene can also be incorporated in a stem cell that produces a substrate (either a cell receptor or enzyme), which can bind with an exogenous probe containing the radiotracer. This allows non-invasive imaging, longitudinal monitoring and study of stem cell biology with high sensitivity. Several

stem cells have been studied using this approach including myocardial stem cell (Fig. 1.4) [32], pancreatic islet cells [33], Thyroid and other organs (using a sodium iodide symporter reporter gene) [33].

1.4 Conclusion

Stem cell transplant techniques are making rapid strides in clinical and research applications especially in newer non-hematological applications. Radiologists have a key role to play in this area as imaging experts and interventionists.

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Chapter 2
Nanotechnology-Based Stem Cell Applications and Imaging

Hanna A. Jensen, Elizabeth M. Martin, Morten O. Jensen, Francesco Romeo, Aldo Di Carlo, Jin-Woo Kim, and Jawahar L. Mehta

Abbreviations

CNT	Carbon nanotubes
DNA	Deoxyribonucleic acid
GNR	Gold nanorods
GNS	Gold nanoshells
GNT	Golden carbon nanotubes
MRI	Magnetic resonance imaging
NIR	Near-infrared
PAI	Photoacoustic imaging
RNA	Ribonucleic acid
SPIO	Superparamagnetic iron oxide

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2.1 Introduction

While the introduction of stem cell-based therapies has significantly widened the horizons of regenerative medicine, notable problems still persist in harvesting the relevant stem cells from the body, introducing them into an optimal microenvironment, and ensuring sustainable differentiation into appropriate and functional tissue once inside the body. Conventional methods of chemically inducing stem cells into specific lineages is being challenged by the advances in biomaterial technology, which suggests that engineered material properties are able to determine stem cell fate [1]. Modern materials such as nanomaterials are designed to conjugate with, or encapsulate the stem cells, to ensure that the artificial microenvironment of transplanted stem cells mimics the chemical and topographical cues that guide differentiation in the extracellular matrix of the native stem cell niche—"convincing" the cells to grow into appropriate, functional tissue. Another useful aspect of joining stem cells with nanomaterials is that nanoparticles can often be imaged with routine clinical imaging modalities such as magnetic resonance imaging (MRI), providing more reliable methods of locating and tracking the transplanted cells.

Potential applications of nanotechnologies in stem cell research include [2]:

- Tracking of stem cell surface molecules and detailed examination of molecular motion without photo-bleaching.
- Noninvasive tracking of stem cells and progenitor cells transplanted in vivo.
- Stem cell delivery systems that enhance the survival of transplanted cells by releasing pro-survival biomolecules.
- Nano-patterned substrates that present covalently tethered biologically active molecules (adhesion sites, growth factors, and synthetic peptides) for stem cell differentiation and transplantation.
- Intracellular delivery of DNA, RNA, proteins, peptides, and small drugs for stem cell differentiation.

In this chapter we aim to present an introduction to nanotechnology and its most explored uses in stem cell applications. Table 2.1 provides a summary of the most common uses and types of nanoparticles in stem cell research, as well as some of their known advantages and disadvantages [2].

2.2 Nanotechnology

The term "nanotechnology" implies that matter is manipulated on an atomic and/or molecular scale. Nobel-prize winners Binnig and Rohrer developed the scanning tunneling microscope in mid-1980's, enabling scientists for the first time to image, measure and manipulate atoms [3]. This led to a nano-revolution that has continued for three decades in the fields of medicine, pharmacology, chemistry, environment,

Table 2.1 The use of common nanoparticles for imaging in stem cell applications. Modified from [66]

Imaging modality	Particles	Comments
MRI	Superparamagnetic Iron Oxide nanoparticles	+ High spatial resolution – Easy to aggregate Non-specific for in-situ labeling Cyto- and tissue toxicity
Optical imaging	Gold nanoparticles	+ Inert character Tunable optical property High spatial and temporal sensitivity – Toxicity
Fluorescent imaging	Quantum dots	+ Tunable emission Photo-stability – Light scattering Cytotoxicity
	Polymer nanoparticles	+ Provide structural support Stable carriers for transplanted stem cells – Photo-bleaching Quenching and leaching
	Silica nanoparticles	+ Enhance the photo-stability of dyes Good ultrasound contrast agents. Easy to merge with other nanomaterials – Potential hemolysis Cytotoxicity

agriculture, household goods, cosmetics, textiles, heavy industry, and more [4]. Nanomedicine is one of the leading fields of nanotechnology and its applied uses extend from diagnostic tools to innovative treatments. Tissue engineering and regeneration has been a major focus of nanotechnology and has led to breakthroughs in tissues such as bone, skin, heart, vessels and bladder [5].

Nanoparticles are 1 nm–100 nm in size and can be shaped as sphere (most common), cube [6, 7], prism [8, 9], hexagon [7, 10], octahedron [11], rod [12, 13], and tube [14]. Morphology and size determine the physicochemical properties of the nanoparticles, as they lead to different cellular uptake and interaction with biological tissues [15].

2.3 Tracking of Nanoparticles In Vivo

The common techniques of imaging of stem cells in vivo have been covered elsewhere in this book. In general, it can be said that the majority of the employed contrast agents often present disadvantages like photo-bleaching over time, interference derived from tissue autofluorescence, chemical and/or metabolic degradation in vivo, and even low transfection efficiency in primary cells and thus are considered suboptimal for in vivo imaging [16]. To overcome this limitation, several engineered nanoparticles with unique magnetic and/or optical properties have been developed and employed in biomedicine, due to their capability to offer real-time methods of tracking intracellular processes at a biomolecular level [17–19]. However, it is important to underline that almost no nanoparticles have been used for stem cell tracking in human patients yet, and thus the bulk of research described is experimental. As particles and technologies develop, so do imaging techniques. We foresee that not only will nanoparticles and stem cell medicine evolve, but imaging technology will also grow to accommodate modern molecular imaging into routine clinical modalities.

The modalities that can currently be utilized for tracking of nanoparticles and conjugated stem cells in vivo are:

2.3.1 Fluorescent Imaging (Light/Confocal/Two-Photon Microscopy)

Several of the nanoparticles can be detected using microscopic optical imaging techniques. Optical imaging is often accessible, of low cost, of high spatial and temporal sensitivity, but lacking in deep tissue penetration. One-, two- and three-photon microscopy has been experimented in imaging for example tumors close to the skin (Fig. 2.1). While currently the penetration depth has a maximum of ~3 mm, scientists are hopeful that it is possible to achieve greater imaging depth by manipulating the fluorescence processes and instrumental set-up [20]. The development of super-resolved fluorescence microscopy by E. Betzig, W.E. Moerner and S. Hell was awarded with the 2014 Nobel Prize.

2.3.2 Magnetic Resonance Imaging (MRI)

Although there are no concerns regarding penetration depth or invasiveness using MRI, the resolution is often insufficient at a molecular and cellular scale, unless contrast agents are employed [16]. Contrast agents usually have a very short lifespan in the body. However, when the contrast agent is incorporated into the cell as a nanoparticle, it greatly enhances the lifespan and allows for long-term tracking capability. A company called BioPAL has developed a gadolinium colloid

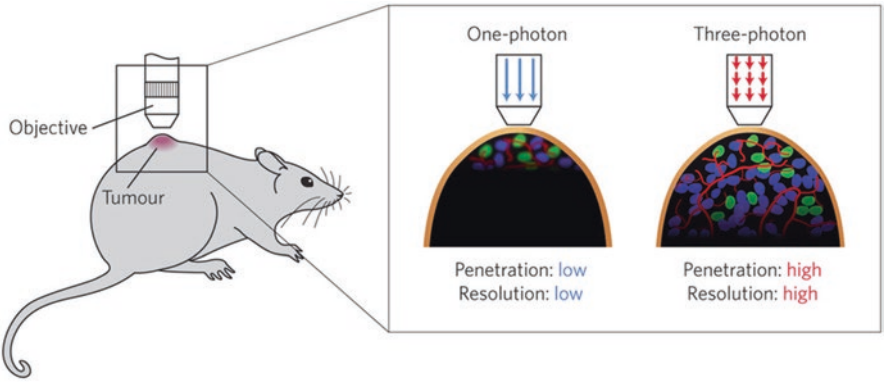

Fig. 2.1 Photon microscopy. Reprinted with permission from Zagorovsky et al. [20]

nanoparticle called Gado CELLTrack™ to locate, track and quantify implanted cells in in vivo using MRI, and thus far this is the only commercially available nanoparticle designed for cell tracking in vivo (only in use for research, no clinical applications at this stage) [21].

In MRI-assisted cell tracking, contrast is achieved through disturbances of the local magnetic field experienced by surrounding hydrogen nuclei. Thus quantification of the number of cells in vivo may be challenging. Furthermore, cell labeling does not necessarily indicate viability of the labelled cell, for example a macrophage can engulf a particle from a dead stem cell and lead to non-specific labeling. Conversely, the contrast may be reduced due to division of stem cells in vivo. Generated contrast can also easily be confounded with other contrast sources such as bleeding or blood vessels [22]. Nevertheless, MRI is being explored as one of the main imaging modalities in nanotechnology-assisted stem cell tracking, and the MRI applicability will be discussed in association with each presented nanoparticle.

2.3.3 Photoacoustic Imaging: Photoacoustic Microscopy and Photoacoustic Tomography

In photoacoustic imaging (PAI), a photoacoustic wave is generated by thermal expansion of tissue after absorption of a short laser pulse [16]. By converting laser into ultrasound emission, PAI combines rich optical contrast, high ultrasonic spatial resolution (100 μm), and deep penetration depth (up to 2 cm) in a single modality, and is becoming an alternative method to fluorescent, MRI, and radioactive imaging for stem cell in vivo tracking [23–27]. It can be combined with ultrasound imaging, and thus has great potential for stem cell therapy and tissue engineering due to non-invasiveness, safety, selectivity, and ability to provide long-term monitoring (Fig. 2.2). PAI has been used successfully in imaging and detecting single nanoparticles in superficial tissues [28–30]. Plasmonic nanoparticles, including gold

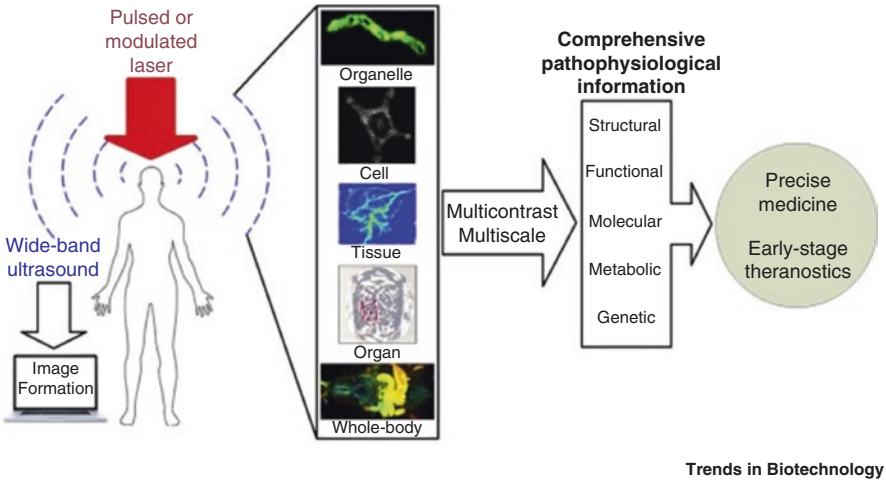

Fig. 2.2 Photoacoustic imaging. Reprinted with permission from Liu et al. [26]

nanoparticles, served as high contrast agents, aiding in PAI of targeted cells [31]. Particularly, tunable near-infrared (NIR)-responsive plasmonic nanoparticles, including gold nanoshells (GNSs), gold nanorods (GNRs), and golden carbon nanotubes (GNTs), have attracted attention for minimally invasive imaging and therapy owing to their high NIR absorption (e.g., ~700–900 nm) in the window of optical transparency of most biological tissues as well as high efficiency conversion of absorbed energy into thermal and acoustic phenomena [28, 31–35]. Although these studies mainly focused on solid and metastatic tumor cells, including stem circulating tumor cells, such plasmonic nanoparticles represent a potentially powerful tool for in vivo PAI of other types of stem cells. Carbon nanotubes (CNTs) are also sensitive contrast nanoagents that are useful for PAI; however, their biocompatibility is in question [28, 30]. This challenge could be overcome by coating CNTs with a thin layer of gold, thereby producing golden nanotubes [33], as well as various types of biocompatible disguising agents [36–39].

In the following section we will outline five types of nanoparticles that have been considered and researched in stem cell medicine, and give an overview of the in vivo imaging that may be applicable.

2.4 Different Nanoparticles and Their Uses in Stem Cell Applications

2.4.1 Superparamagnetic Iron Oxide Nanoparticles

Superparamagnetic Iron Oxide nanoparticles or SPIOs are the only commercial nanoparticles that have been utilized in clinical applications. In 2009, the Food and Drug Administration approved ferumoxides (Feridex®) and ferucarbotran (Resovist©)

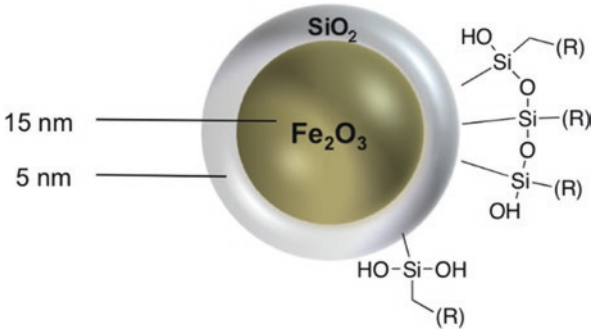

Fig. 2.3 Silica-coated iron oxide nanoparticle. Reprinted with open-access permission from Bohmer et al. [40]

for clinical use as liver-specific contrast agents—however, the production of both has since been ceased due to commercial reasons. SPIOs have an iron-oxide core (usually of magnetite or maghemite), a coating layer (often dextran or carboxydextran, chitosan, silica or gelatin) that stabilizes the magnetic core, and finally surface functional groups (such as polyethylene glycol and polystyrene) that provide hydrophilicity and stability, and prevent nanoparticle aggregation (Fig. 2.3). SPIOs act as good T-2 contrast agents in MRI, enhancing the contrast between tissues [16].

There has been marked variety in the composition of SPIOs including different types of iron cores, different coating layers, different transfection agents (peptides helping SPIO incorporation into the cell), and significantly differing dosing strategies across published studies, precluding robust conclusions about their usage in stem cell applications thus far. However, mesenchymal stem cells, adipose-derived stem cells and neural progenitor cells have been labeled and tracked with SPIOs in vivo in a rabbit [41] and a rat [42], in diseases such as cardiovascular disease, skeletal tissue injury, traumatic brain injury, stroke, spinal cord injury, and multiple sclerosis [43–49]. Beyond tracking transplanted stem cells, SPIOs have also been utilized in identifying and labeling endogenous stem cells in vivo [50]. Most of these studies report that more than 90% of cells contained enough iron to allow for their detection without significant alterations in cell viability and differentiation, however reports do exist of decreased cell proliferation and migration, as well as signs of inflammation [51].

In clinical trials involving bone marrow derived stem cells and hematopoietic stem cells that are used in patients within 24 h after their isolation, the labeling of stem cells with SPIO nanoparticles should be performed in less than one day. A rapid method to label stem cells has been reported based in the electroporation of cells (“magnetoelectroporation”) [52]. This technique involves low-voltage pulses to induce endocytosis of contrast agents in a matter of minutes. In addition to the advantage of rapid labeling of cells, this technique does not require transfection agents for the internalization of SPIO nanoparticles, which simplifies the regulatory pathway required for approval by regulatory agencies.

A number of factors affect the MRI detection threshold of SPIOs-labeled cells, such as the SPIO concentration per cell, and intrinsic MRI parameters, such as field strength, signal-to noise ratio, pulse sequence, and acquisition parameters. In most

cases MRI detection requires clusters of thousands of labeled cells [47], and this becomes problematic as extensive in vivo-migration of SPIOs labelled cells occurs and density of cells in a given area is reduced over time. Long-term observation of SPIOs labelled stem cells may also be limited because of dilution by cell division [2]. Thus further research and eventual standardization of SPIOs is needed before the marked potential in becoming a routine method of stem cell labeling and in vivo tracking via MRI is brought to realization.

2.4.2 Quantum Dots

Quantum dots are spherically shaped semiconductor light-emitting crystals with a diameter of approximately 2–10 nm (Fig. 2.4). They have the ability to convert short wavelength light into nearly any color in the visible spectrum with a high efficiency [53]. The general public may recognize quantum dots as their properties are currently exploited in the electronics industry in making brighter television screens.

Quantum dots have a solid long-term photo-stability and durability which makes them ideal for live-cell imaging and dynamics studies. They can also concurrently tag multiple inter- and intracellular components for time ranging from seconds to months. Thanks to the narrow emission spectrum and broad excitation spectrum of quantum dots, several cell components can be visualized with fluorescent microscopy by using different colored quantum dots in vivo [2, 54]. Based on these favorable properties, quantum dots have been used for almost two decades for bio-imaging applications, in particular, to label different cell lines for both in vitro and in vivo studies [55, 56]. Common methods used for an efficient intracellular delivery of quantum dots are microinjection, electroporation, lipid based transduction, and peptide-mediated delivery [16].

Quantum dots consist of elements such as indium phosphamide and cadmium telluride, the latter being extremely toxic to humans. Investigators have aimed to mitigate this problem by coating the cadmium core of a quantum dot with a zinc sulfide buffer layer or manufacturing cadmium-free quantum dots [57–59]. In experimental in vitro studies done in human embryonic and mesenchymal stem cells, quantum dots have been shown to be mostly safe without major interferences in stem cell morphology, viability, proliferation or differentiation [60–62]. In vivo preclinical studies have shown that quantum dot-labeled stem cells can be tracked in the mammalian nervous system (neural stem cells and neural progenitor cells) [63], cardiac tissue (mesenchymal stem cells) [64] and in angiogenesis (embryonic stem cells) [65].

Quantum dots are most appropriate for fluorescence imaging, and the main challenge is light scattering that makes it difficult to locate the labelled cells in 3D and to estimate cell survival in quantity [66]. Quantum dots are suitable for in vivo imaging with: fluorescence (light/confocal/two-photon microscopy).

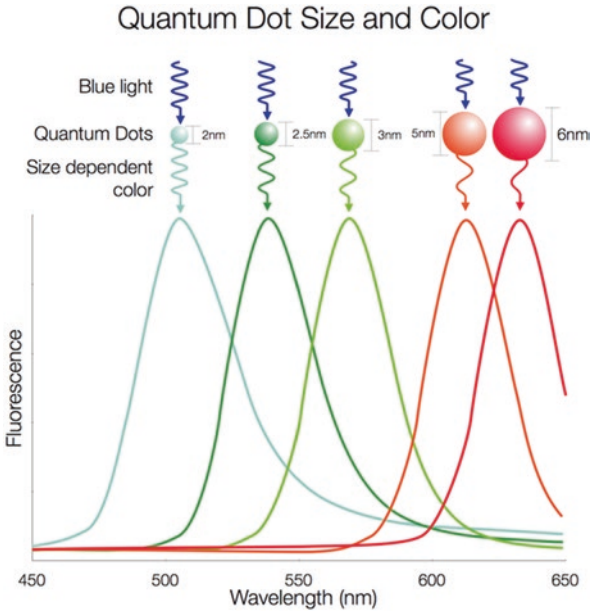

Fig. 2.4 Quantum dot size and color. Picture courtesy of Nanosys Inc., reprinted with permission

2.4.3 Silica Nanoparticles

Silica nanoparticles do not have autofluorescence, but rather act as a matrix to chemically and physically confine organic dye molecules for fluorescence imaging [16]. Dye molecules are encapsulated within a silica shell, protected from external quenchers. The silica capsule can enhance the photo-stability of the dye and provide a biocompatible surface for bioconjugation [67]. Silica as a material is mostly used for drug delivery [68] and have been explored as a transplantation vehicle for stem cells as they provide large surfaces for stem cells to adhere, grow and mature [66]. Thus far there have been no experiments of imaging silica nanoparticles in vivo. However, reports exist of human mesenchymal stem cells internalizing silica particles conjugated with fluorescein isothiocyanate [69] and cyanine [70] in vitro. In these experiments silica nanoparticles did not affect the viability, proliferation, and surface marker expression or differentiation capabilities of the stem cells. Silica nanoparticles can be useful as ultrasound contrast agents [71]. They have also been combined with other nanomaterials to enhance biocompatibility and cellular uptake; examples include silica-coated gold nanoparticles [72], magnetic nanoparticles [32] and quantum dots [73], in which the silica shell enhances the photoacoustic signals generated by nanoparticles (Fig. 2.5).

Silica nanoparticles are most suitable for in vivo imaging with fluorescence imaging.

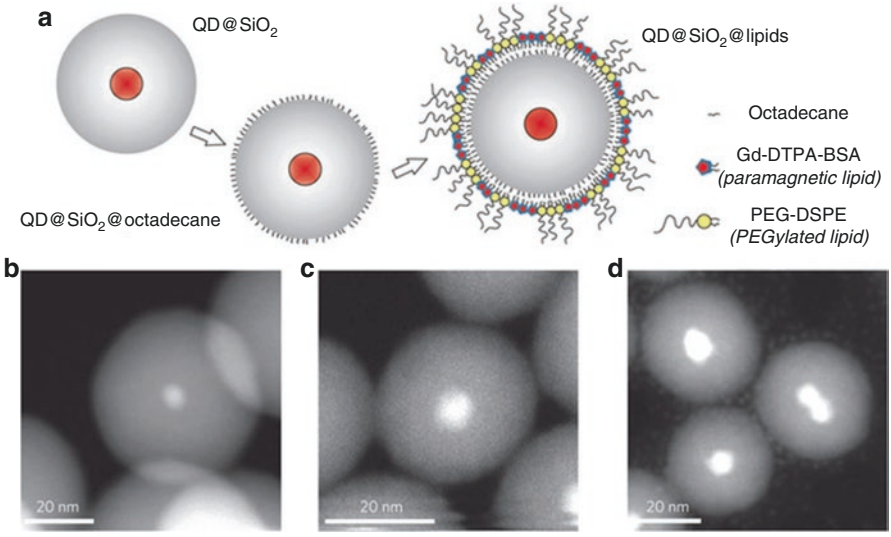

Fig. 2.5 Overview of the intermediate steps involved in the synthesis of lipid-coated, quantum dot (QD)-containing silica nanoparticles. Silica nanoparticles are first made hydrophobic by covalently attaching octadecane chains to the silica surface. Subsequently, paramagnetic Gd–DTPA–DSA and poly(ethylene glycol)-containing PEG–DSPE lipids are applied to coat the hydrophobic QD@SiO2 nanoparticles with a monolayer of lipids. This nanoparticle platform serves as a bioapplicable, multimodal contrast agent for MRI and fluorescent techniques. Reprinted with permission from van Schooneveld et al. [73]

2.4.4 Polymer Nanoparticles

Polymer nanoparticles can be used in stem cell tracking when conjugated with fluorescent organic dyes. The dye can be entrapped in the polymer interior (similarly to silica nanoparticles), or covalently bound to the polymer chain before the nanoparticle is prepared by dispersion of preformed polymers or polymerization of monomers [16]. Polymer nanoparticles can be formed as dendrimers, microgels and modified polysaccharide nanoparticles, however the most common fluorescent polymer nanoparticle is the polystyrene nanoparticle (Fig. 2.6).

Studies have shown that mesenchymal stem cells uptake polystyrene nanoparticles, however the stability of the dye molecules is sub-optimal with clear evidence of photo-bleaching, quenching and leaching [74]—thus polymer nanoparticles still have some way to go before being applicable to human long-term stem cell tracking. They have, however, demonstrated an aptitude for delivering genes and proteins into stem cells, in acting as carriers for transplanted stem cells, as well as drug delivery using stem cells as vehicles [66]. Polymers can provide structural support for stem cells that are introduced to the body, and control the biomolecules released for modulation of stem cells in vivo [75, 76]

Polymer nanoparticles are most suitable for in vivo imaging with fluorescence imaging.

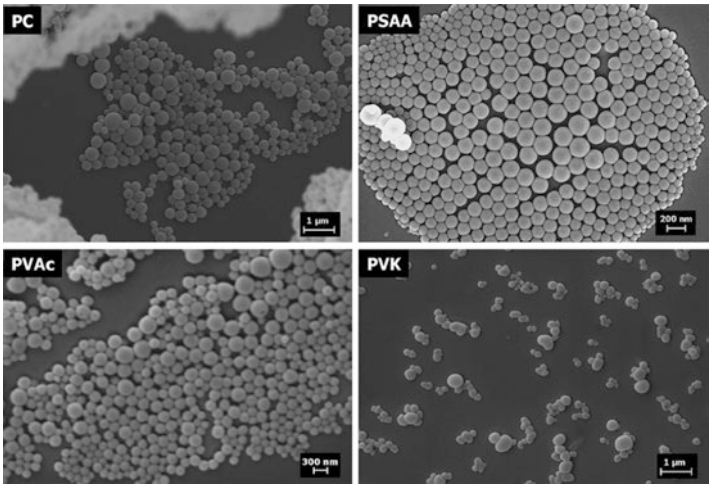

Fig. 2.6 Scanning Electron Microscopy images of polymeric nanoparticles of poly(carbonate) (PC), poly(styrene-co-acrylic acid) (PS-co-AA), poly(vinyl acetate) (PVAc) and poly(vinyl carbazole) (PVK) prepared by dialysis of the polymers. Reprinted with permission from Hornig et al. [77]

2.4.5 Gold Nanoparticles

Gold nanoparticles (Fig. 2.7) have several properties that make them a promising agent for stem cell labeling and tracking. They have a strong "surface plasmon resonance" i.e. resonant oscillation of conduction electrons at an interface stimulated by light; this means that they can easily scatter and adsorb visible light. They have a high spatial and temporal sensitivity compared to MRI, positron emission tomography (PET) and computer tomography (CT) [78]. It is also relatively easy to manufacture gold nanoparticles and conjugate them to a variety of biomolecular targets [16]. Gold nanoparticles can also be induced by infrared light, which causes vibrational energy and heat that can for example kill select cancer cells [79]. In particular, gold nanoparticles and their hybrids with different shapes, including GNSs, GNRs, and GNTs that were tuned to desired NIR spectral ranges, have shown their potential for biomedical photoacoustic imaging and photothermal therapy. The optical tunability and responsiveness in the NIR range (i.e., 650 to 1400 nm) offer promising potential for minimally invasive theranostics of diseases because of the relative transparency of biological tissues to NIR [27, 28, 30–33, 35, 80]. Most biological components are relatively transparent to NIR. Also NIR responsive nanoparticles allow selective and sensitive sensing of targets in the presence of biological background materials, minimizing the sample preparation and purification time. Furthermore, as discussed above with, coating the gold nanoparticles with a silica layer facilitates the uptake to cell and increases their photoacoustic signal [25].

For stem cell tracking, most studies have utilized GNRs [81]. Studies have demonstrated their successful mesenchymal cell labeling and photoacoustic longitudinal in vivo tracking in mice and rats, with results validated by histology and mass

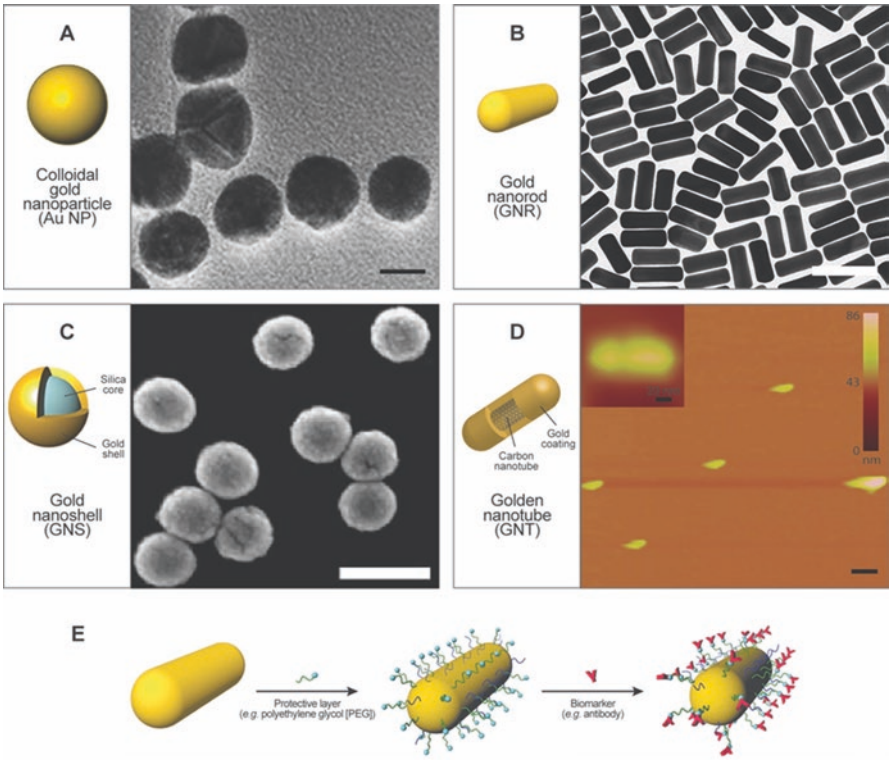

Fig. 2.7 Plasmon-resonant gold nanoparticles in biomedical applications: (**a**) colloidal gold nanoparticles; (**b**) gold nanorods (GNR) [85]; (**c**) gold nanoshells (GNS) [86, 87]; (**d**) golden carbon nanotubes (GNT) [33]; (**e**) bioconjugation of gold nanoparticles using GNR as an example [88]. Scale bars represent: 10 nm (**a**), 100 nm (**b**), 300 nm (**c**), and 100 nm (**d**). Adapted from the data of the cited articles by permission from American Chemical Society, Biomedical Engineering Society, Wiley, Nature Publishing Group, and American Scientific Publishers. Note that some of original images were amended by cropping, re-coloring, and rotating them

spectrometry analysis [24, 25]. In vitro studies have shown that mesenchymal and adipose-derived stem cells labeled with gold nanoparticles did not demonstrate changes in cell function or proliferation, and no toxicity was apparent [23, 25]. However, it is hypothesized that the intracellular biocompatibility of gold nanoparticles is influenced by the presence of free radicals which can lead to oxidative stress and cell damage [66]. Also, before the translation of nanoparticles to clinical practice, there is a need to address issues like opsonization, phagocytosis by macrophages, and sequestration to the liver and spleen for eventual elimination from the body, which will eventually determine the particle's longevity in circulation and clearance rate from the body [29, 36]. The fates of foreign nanoparticles and their hybrid nanoconstructs in vivo depend upon their physico-biochemical properties, including their size, shape, and surface chemistry. Some novel approaches have

been reported to engineer nanoparticles and their architectures with desirable size, shape, and functionality, including those based on the "nano-toolbox"-based programmable self-assembly approach [29, 36, 82–84]. The capability of modulating the geometric configurations and surface characteristics suggests opportunities to overcome the hurdle, while considerably increasing blood circulation times as well as biocompatibility [29, 36, 82].

2.5 Nanoenvironments and Nano-Scaffolds

In living organisms, stem cells are prevented from exiting the mitotic cycle by specific environments, called niches [89]. These niches are formed by cellular and non-cellular elements. The non-cellular elements include instructive extracellular matrix molecules (e.g., collagen, elastin, proteoglycan, fibronectin, and laminin) secreted by cells in the vicinity of stem cells. The nanoscale structure of the extracellular matrix provides cellular anchorage points and presents clues to guide cell behavior. The ability to engineer materials to resemble the structural complexity of the extracellular matrix, including its nano-textured topography, has made large contributions to our understanding of several cellular processes including stem cell-matrix interactions, stem cell differentiation in response to different nanoscale topographies, and stem cell migration [2]. Tissue engineered nano-scaffolds can assist in cell adhesion, engraftment survival, migration, differentiation, and organization. These nano-scaffolds often containing nanofibers have been shown to improve mesenchymal stem cell viability [90]. One of the ultimate applications of nanofiber-based scaffolds is in vivo stem cell transplantation, where nano-scaffold would act as a temporary extracellular matrix to guide tissue formation and typically would degrade in concert with deposition of new in vivo matrix. Unfortunately, there are few in vivo studies of stem cells transplanted into these scaffolds [91]. In contrast to traditional scaffolds for cell transplantation, nanofiber-based scaffolds offer the opportunity to control stem cell behavior by incorporation of high-density epitopes and control of cell alignment. Moreover, the intrinsic properties of the scaffolds might contribute to the differentiation of endogenous stem cells in the vicinity of the implant.

In Fig. 2.8 below stem cells or progenitor cells are seeded on three-dimensional scaffolds formed by nanofibers. These nanofibers may present a high density of ligands, including cell-adhesion epitopes or immobilized growth factors, for stem cell differentiation. The tissue constructs can be implanted immediately after incorporation of a cell source (<24 h) into the defective tissue. Alternatively, the tissue constructs can be cultured in bioreactors to allow cell proliferation, differentiation, and three-dimensional organization before their final implantation. In both cases, the scaffold acts as a temporary 3D ECM for cell adhesion and tissue formation and typically is designed to degrade when new extracellular matrix is deposited [2].

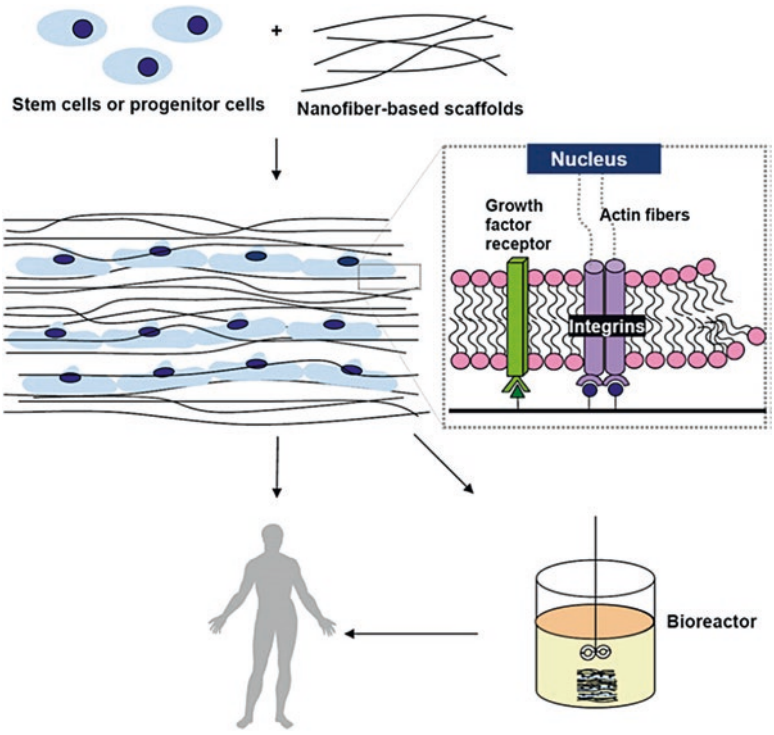

Fig. 2.8 Example of a nano-scaffold. Reprinted with permission from Ferreira et al. [2]

2.6 General Safety

Due to exponentially growing numbers of suggested nanotechnology applications, a branch of science has been born to specifically address the adverse effects caused by nanomaterials. Nanotoxicology studies nanoparticle-induced toxicity in in vitro as well as in vivo experimental models in order to contribute to the development of a sustainable and safe nanotechnology [92]. It aims to optimize well known toxicity tests or produce new ones to be applicable for nanosafety evaluation [93, 94]. Over the last years, research groups that study the outcomes of nanoparticles used for stem cell tracking are focusing on their possible undesirable effects inside the experimental model or even the host. Thus, prior to the therapeutic use of nanoparticles, it is becoming increasingly important to conduct systematic in vitro studies to assess their toxicological profiles and evaluate their potential influence on the self-renewal and differentiation properties of stem cells [2, 16, 95].

2.7 Conclusions

With over 2000 clinical trials involving stem cells underway, it is critical that methods to deliver and track stem cells in vivo are optimized. While nanotechnology can be of significant assistance, there are important concerns that need to be addressed prior to mainstream clinical applications. The loss of contrast of nanoparticles as stem cells proliferate and differentiate in vivo, as well as the low efficiency and specificity of in situ labeling of endogenous stem cells are areas of great interest and challenge. Beyond tracking cells, it is of extreme importance to determine whether stem cells are viable and have differentiated into functional target tissue, and at this stage this often requires a variety of complementary imaging techniques. Toxicity of magnetic and metal particles is still unclear and requires thorough long-term preclinical testing. Nevertheless, as technologies and techniques advance, biomaterials will likely be essential in delivery, assurance of viability, labelling and tracking of stem cells in a variety of medical applications.

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Chapter 3
Radiologic Procedures Used in Pediatric Stem Cell Transplantation

Mohammad Atiq, Terry Harville, Nishi Shah, Paulette Mehta, Tarun Pandey, and Yogesh S. Jethava

3.1 Introduction

Radiologic procedures form one of the cornerstones of diagnosis in hematological malignancies. Various radiological procedures are essential for the diagnosis, assessment of the therapeutic response, to identify disease relapse, and to evaluate for complications pre- and post-transplantation. The significance of radiology and radiological procedures in stem cell transplantation is expected to grow. In this chapter, we discuss the radiologic procedures and review the key imaging modalities and implications related to pediatric hematopoietic stem cell transplant (HSCT). We will also discuss the role of radionuclide Imaging for patients undergoing hematopoietic stem cell transplantation with a brief review of use of radio-labeled MIBG imaging in children with Neuroblastoma.

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3.2 Imaging Modalities in Stem Cell Transplant

3.2.1 Computed Tomography

After HSCT, there are many known complications that physicians must keep in mind. The timeline of each occurrence helps to narrow the focus on the pathogenic mechanism responsible. While clinical presentation is the guiding principle by which physicians must approach potential complications, the importance of radiologic procedures in the diagnosis and follow up cannot be overstated. Computed Tomography (CT) plays a major role in assessing potential complications after HSCT. In the post transplant setting, one can categorize complications based on timeline, as well as, the affected organ systems.

More than 50% of pediatric HSCT patients experience pulmonary complications, which can be infectious or non-infectious. Bacteria or Fungi cause some of the early infectious complications. While plain radiographs have utility in identifying consolidative changes, chest CT is much more useful for infections with atypical features. It is very helpful for identifying fungal infections, such as with invasive aspergillosis, in which chest radiographs may demonstrate normal appearance, but the chest CT may show airspace opacification, pulmonary nodules, air crescent or "halo" sign, or cavitary lesions (Fig. 3.1). Tracheobronchial aspergillosis is also well visualized on

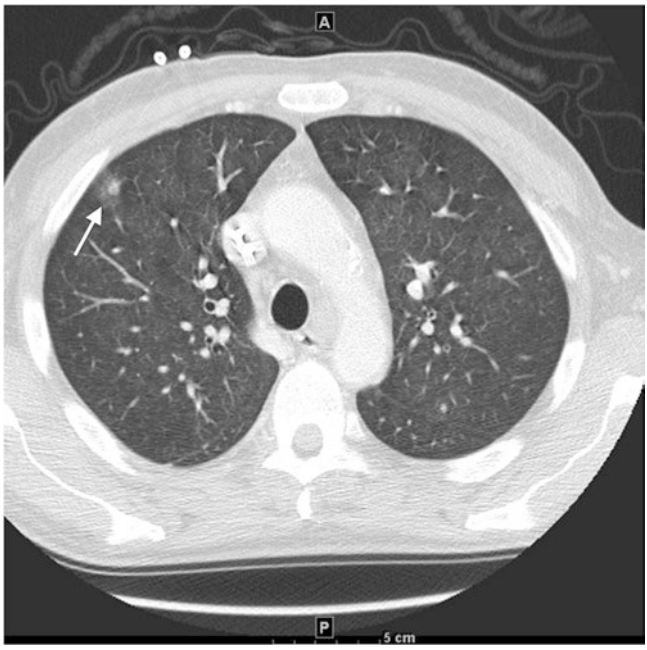

Fig. 3.1 Aspergillus pneumonia after allogenic transplant. Classic but nonspecific 'halo sign', with nodules surrounded by ground-glass opacity (*arrow*)

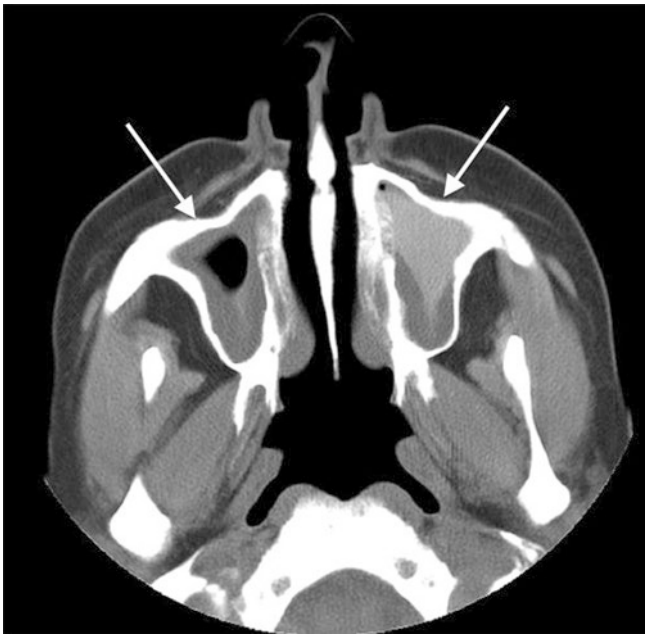

Fig. 3.2 Zygomycetes sinusitis 1 year post-transplant. Axial CT image through the maxillary antra demonstrate bilateral mucosal thickening with high attenuation material within the opacified left maxillary antrum, suggesting fungal infection

CT with thickened airway walls, debris within the airway, and sparse peribronchial consolidation or small centrilobular nodules [1]. Chest CT has utility in identifying CMV pneumonia which is generally bilateral with poorly defined nodules and patchy, diffuse ground-glass attenuation. Paranasal sinusitis is a complication in which CT imaging is very helpful and is even advocated by some to be used as a screening tool. Usually, air-fluid levels and soft-tissue thickening are present on CT imaging (Fig. 3.2). Direct coronal CT is the preferred imaging technique as it is more sensitive for detecting the more understated changes that could be of significant importance in the management of this disease. This is very important in the case of invasive fungal sinusitis as it is generally rapidly progressive and can have dire consequences [2].

Later complications include bronchiolitis obliterans and cryptogenic organizing pneumonia. Bronchiolitis obliterans is a unique long-term complication after allogeneic stem cell transplantation; plain radiographs may show some abnormalities, but children can have severe nonreversible obstructive abnormalities detected by pulmonary function testing, when the chest X-ray may be interpreted as near-normal. Chest CT is of a higher diagnostic yield and may show a mosaic pattern (where parenchymal lucencies are seen adjacent to normal lung), expiratory air trapping, as well as, bronchial dilation and bronchial wall thickening (Fig. 3.3) [1]. Cryptogenic organizing pneumonia would be seen as sparse peripheral infiltrates, usually peribronchial or subpleural on chest CT, with restrictive pattern seen on the pulmonary function tests [3].

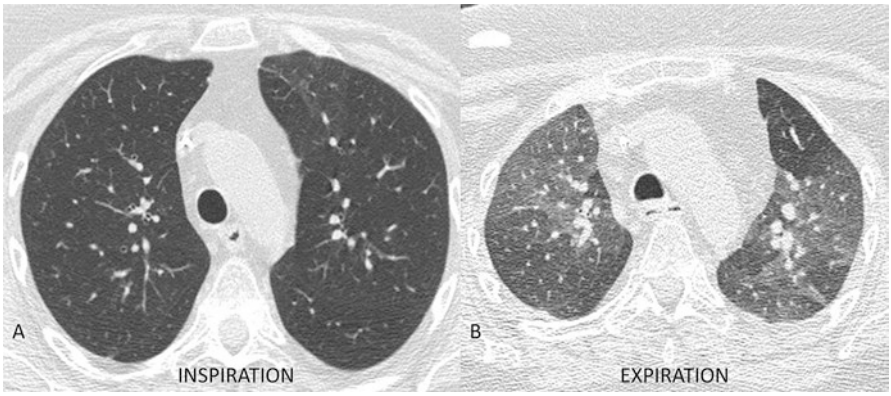

Fig. 3.3 Bronchiolitis obliterans post stem cell transplant. Inspiration (**a**) and expiration (**b**) axial CT images at the level of the upper lobes demonstrate areas of air-trapping

Abdominal and gastrointestinal complications are common in patients post-HSCT, with some being found in specific subsets of patients. A variety of abdominal complications can have overlapping clinical presentations. Appropriate imaging makes it much easier to differentiate between them, especially when combined with the clinical picture. Radiation enteritis generally presents as small bowel wall thickening with inflammatory changes in the fat of the adjacent mesentery and retroperitoneum on CT. Colitis, due to either infectious causes or GVHD, may have similar clinical presentations, but may have certain identifying features on CT that could help distinguish a more distal disease from neutropenic entercolitis (typhilitis), which is typically found in the cecum, and is rarely seen in adults, but more common in the pediatric population (Fig. 3.4) [4]. It may manifest as cecal wall thickening with signs of inflammation in the surrounding fat and free intraabdominal fluid. If the colitis advances to perforation then free intra-abdominal air would be seen. Pseudomembranous colitis shows significant colonic wall thickening (11–15 mm) that is uncommon in other types of colitis, mucosal enhancement in the affected areas, and low-attenuation of the haustral folds. One sign particularly indicative of pseudomembranous colitis is the "accordion sign" which is due to the infiltration of contrast material between the thickened colonic mucosa and the pseudomembranes (Fig. 3.5) [4]. Pneumatosis intestinalis is a complication of HSCT that can be secondary to many factors and may be a benign or serious finding depending on the patient's presentation [1]. It can be described as having "bubbly" and "linear" intramural lucencies i.e., the detection of gas in the intestinal walls. Pneumatosis intestinalis, if found with concurrent neutropenic colitis, signals risks for impending bowel perforation.

Acute GVHD has a very good prognosis when treated quickly. The small bowel and colon are among the earliest and most common tissues affected. Abdominal manifestations on CT imaging typically demonstrate diffuse small bowel and colonic wall enhancement with adjacent inflammatory changes, which when widespread, can lead to infiltration of mesenteric fat that will show up as mesenteric stranding. Bowel wall thickening is not very sensitive or specific for GVHD, and

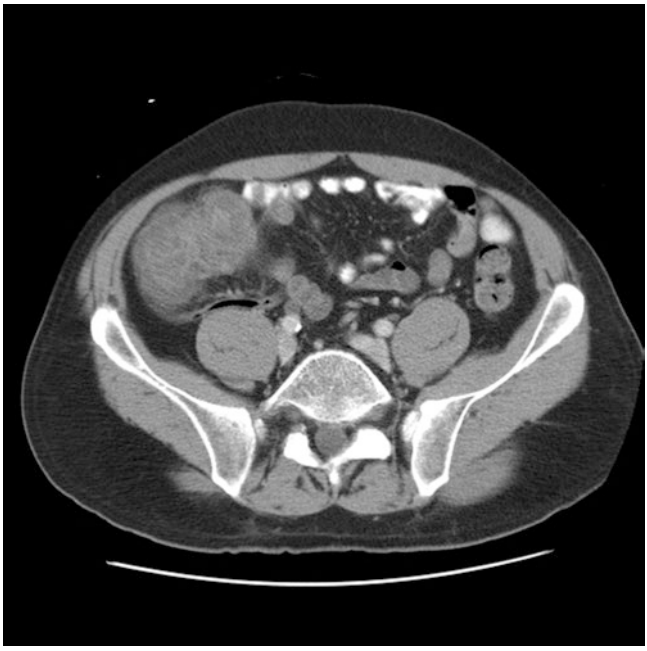

Fig. 3.4 Axial CT image in a patient with history of AML with lower abd pain, diarrhea and neutropenia (wbc = 0.2) post transplant. Notice marked thickening of the cecum and fat stranding. Diagnosis: Neutropenic colitis

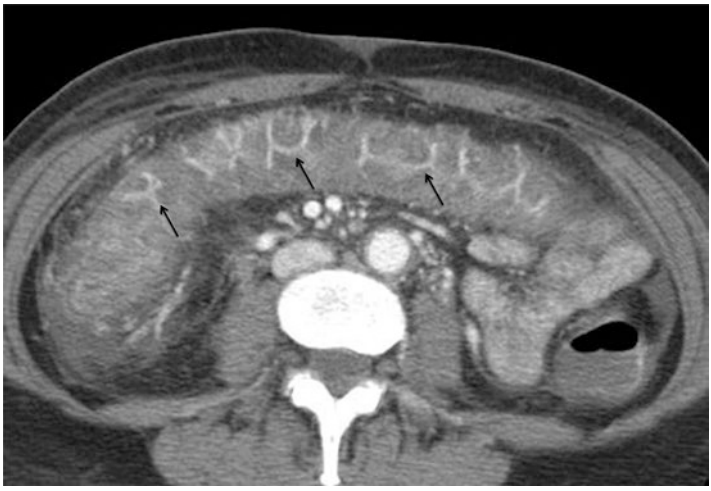

Fig. 3.5 Axial post contrast CT image from a patient with history of AML who presents with diarrhea post transplant. Notice markedly edematous transverse colon mucosa with minimal contrast entrapped between the edematous haustra (*arrows*), also called as the accordion sign

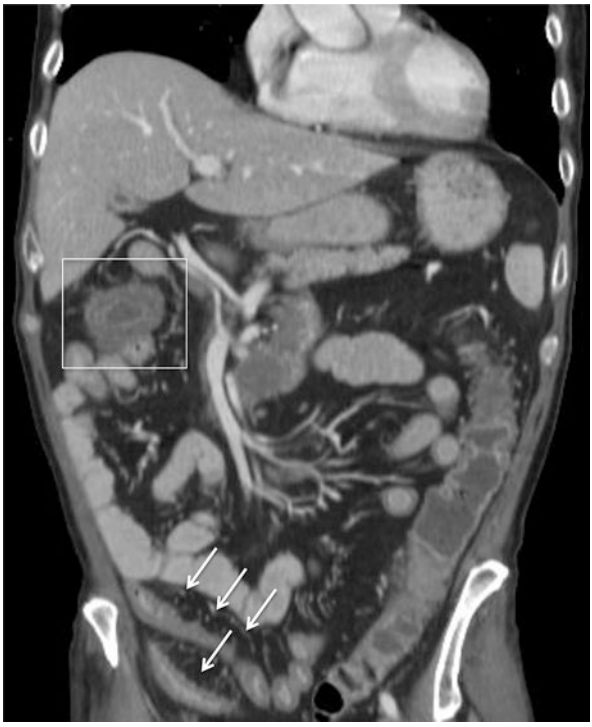

Fig. 3.6 Coronal post contrast CT from a case with proven graft versus host disease (GVHD). Patient presented with abd pain, diarrhea and bloating post transplant for NHL. Notice the thickened large bowel loops with hyperemia (*arrows*) in mesenteric vessels (*Coomb sign*). Also seen is the Halo sign (*box*) around a loop of inflamed colon

can be seen in other inflammatory conditions affecting the intestines, e.g., CMV infection and enterocolitis. Moreover, in GVHD, it can be present in varying degrees, but as compared to small bowel follow through contrast radiographic studies, CT has the advantage of a better image of luminal and extra luminal disease and does not necessitate the use of oral contrast, which may be difficult to tolerate in some of the patients in the pediatric population [1]. The bowel wall itself may manifest in a radiologic sign that is known as the "halo sign", which is due to hyperemic granulation tissue surrounded by lower-attenuation outer bowel wall layers [3]. An additional finding that may be present is the "comb sign" which is due to engorged blood vessels (Fig. 3.6) [4].

Liver abnormalities and complications are common in post-HSCT patients. One unfortunately too frequent hepatic complication in the peri-transplant period is veno-occlusive disease. Multidetector CT will demonstrate diffuse, hypoattenuating liver parenchyma, ascites, periportal and gallbladder wall edema, and possibly narrowed hepatic veins, therefore being useful for excluding other causes of the symptoms. Hepatic fungal or bacterial abscesses are also seen in patients post HSCT. A contrast-enhanced CT or MRI may be superior to ultrasonography, specifically for detection of fungal lesions (Fig. 3.7). CT findings include small hypoattenuating lesions with peripheral ring enhancement, and detection of these lesions is improved when CT imaging is collected during both the arterial phase and venous phase [4]. Genitourinary complications can include hemorrhagic cystitis, renal abscesses, and renal parenchymal loss. Hemorrhagic cystitis can be seen after treatment with

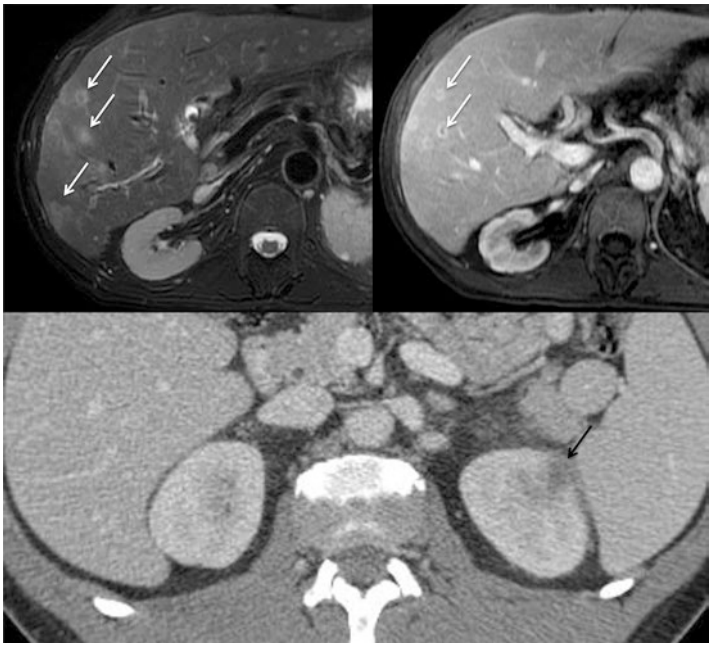

Fig. 3.7 Hepatic micro abscesses (*top*) and left renal abscess in two different patients following HSCT. Axial post contrast T2 W fat sat images through the liver (*Top Left*) and post contrast T1 W images (*right*) show multiple T2 hyperintense liver lesions that show ring like enhancement on post contrast images (*arrows*). Axial post contrast CT image shows a hypodense non enhancing lesion near the superior pole of the left kidney compatible with renal abscess (*black arrow, bottom image*)

cyclophosphamide and shows up on CT as focal or diffuse bladder wall thickening, intraluminal clots, and sloughed mucosa. Renal abscesses will show up as hypoattenuating collections, with wall enhancement on contrast-enhanced CT (Fig. 3.7). Renal parenchymal loss can be seen in patients who have received radiation therapy and manifest on CT as atrophy of the kidney [2].

Although HSCT is performed for both malignant and non-malignant diseases, there is always a chance that patients may develop secondary malignancies following the preparative regimens. These include post-transplantation lymphoproliferative disorder (PTLD), lymphoma, leukemia, myelodysplasia, or various solid tumors. For PTLD, it is recommended that chest, abdomen, and pelvis CT be utilized for staging purposes. Findings on imaging may include lymphadenopathy, hepatosplenomegaly, focal parenchymal masses and nodules, and diffuse organ infiltration without a focal mass [2]. CT with contrast material enhancement is the preferred radiologic procedure for assessing the degree of lymphadenopathy and/or organ involvement in patients in whom there is post-transplantation lymphoproliferative disorder (PTLD) or lymphoma [1]. It is also important to note that patients under that age of 10 have the greatest overall risk of having post-transplantation development of malignancy [4].

Although cerebrovascular events are rare post-stem cell transplantation, early assessment via head CT to differentiate between hemorrhage and infarction is clini-

cally important for preventing further morbidity or mortality of these serious complications. Subdural hematoma, is one of the most common cerebrovascular complications post-HSCT detected by CT. Thus CT scan is a rapid modality of radiological procedures which gives immediate answers for appropriate treatment decisions.

3.2.2 Positron Emission Tomography (PET)/Magnetic Resonance Imaging (MR)

The roles of Positron Emission Tomography and Magnetic Resonance Imaging are critical in the late post-transplantation period (>100 days) when complications such as avascular necrosis and secondary malignancies become more important, but both radiographic technologies are of significance earlier as well [1, 5, 6].

Central Nervous System (CNS) infections can be caused by a variety of organisms with findings that can be well demonstrated on MR, such as enlargement of the ventricular or subarachnoid spaces in opportunistic infections, and abscesses or granulomas with or without ring enhancement. *Aspergillosis* seen on T2-weighted MR images can show areas of intermediate signal intensity that have peripheral rings of hyperintensity. It is important to note that ring-like enhancement correlates with a less invasive form of disease, suggesting host immune response. Herpes virus infections may also be diagnosed on MR. Herpes encephalitis can lead to white matter demyelination, as well as, changes in signal intensity of the temporal lobes. In the case of HHV-6 infection, MR shows increased temporal lobe signal intensity on T2-weighted images and increased hippocampal glucose uptake on PET scan. Epstein Barr Virus (EBV) infection has been demonstrated on MR as focal infarcts with rim-like enhancement.

Toxicity from immunosuppressive therapies, such as cyclosporine, may present as posterior reversible encephalopathy syndrome (PRES), which on MR imaging of the brain shows up as symmetrically distributed areas of vasogenic edema in the areas supplied by the posterior circulation (Fig. 3.8). Other findings include focal gray and white matter changes, watershed vascular injuries, and/or central pontine myelinolysis. This differs from the MR findings seen in patients with encephalopathy due to the conditioning regimen prior to transplant and/or radiation therapy, as one will usually see widespread focal deep white matter and periventricular white matter changes, leukoencephalopathy, and diffuse cerebral atrophy depending on the time elapsed since HSCT [1].

After HSCT, bone mineral density is decreased. This combined with a multitude of other factors, including but not limited to corticosteroid exposure, irradiation, and inactivity combine to place the transplant recipient at risk for avascular necrosis, particularly of the hips and knees. While a plain radiograph is likely to be the first test obtained in assessing for avascular necrosis, MR is more sensitive and is endorsed for yearly screening in this at-risk population [1]. As MR can lead to earlier detection, it also allows for earlier intervention in the patients in whom the beginning signs of avascular necrosis are detected. Avascular necrosis on MR shows up as heterogeneous signal intensity changes that may involve collapse of

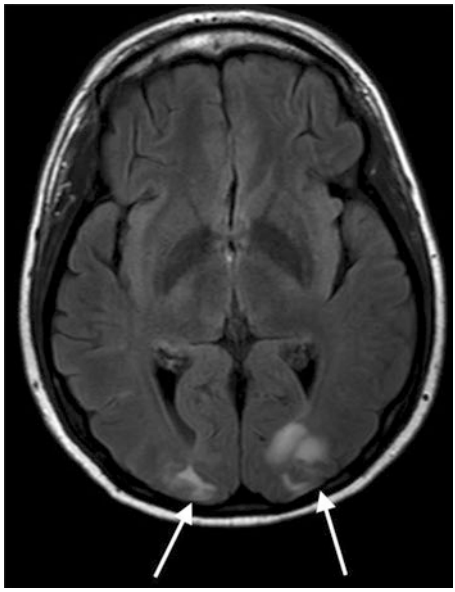

Fig. 3.8 Posterior reversible encephalopathy syndrome (PRES) 6 weeks after transplantation. Axial FLAIR image shows occipital hyperintensity in the typical distribution of PRES (*arrows*)

the articular surface. In addition, patients may develop bone infarction, which is seen as various areas of abnormal signal intensity within the bone on MR [2].

3.2.3 Radionuclide Imaging for Patients Undergoing Hematopoietic Stem Cell Transplantation (HSCT)

Unfortunately, there is scant literature describing the use of radionuclide imaging for localization of infections in patients who are preparing to undergoing HSCT. Primarily, articles describe the use in the initial diagnosis and for other assessment of the disease status, or there are text book descriptions and illustrations of specific instances of use [7–9]. In the past though, radionuclide imaging was an important step in identifying potential infections in children with immunodeficiencies prior to HSCT. Localization and appropriate treatment was necessary to attain success with HSCT. If a hidden infection was present, and the child received myeloablative chemotherapy, severe life-threatening infectious complications could ensue. The standard approach would be to perform a Technetium-99 m or Gallium 67 scan for localization of sites of inflammation and potential infection. Occasionally, these would be performed in tandem, a few days apart, to allow the previous radionuclide to decay. Alternatively, a "tagged-white cell scan" (indium-111 scan) would be used, with the patient's "tagged-neutrophils" migrating to the potential site of infection. Initially, the radioactive emissions would be captured on 2-dimensional radiographic film or on a scanner plate. The potential site of inflammation or infection could be approximately located, and then a subsequent ultrasound or CT scan

could be used to better define the location or be used directly to assist with aspiration or biopsy of the area. Stains and culture of the obtained material could lead to appropriate antimicrobial treatment with expected better subsequent outcomes with the HSCT. The radionuclide assays were improved during the 1980s, as computerization improved, and MR imaging became available. The information captured on the scanner plate could be superimposed on the CT scan or MR image in a 3-dimensional fashion, allowing for better colocalization of the putative site of infection, thus resulting in easier attempts for aspiration or biopsy of the sites. Currently, PET scanning or SPECT scanning have more-or-less supplanted the previously used radionuclide scans, since greater resolution can be achieved and better localization of a potential infected site can be found [10].

In summary for patients with immunodeficiency preparing to undergo HSCT there is risk for hidden infection to be present, especially if neutropenia is present, or fevers have been occurring. Identification of a site of potential infection with the most appropriate currently available imaging procedure, and then using aspiration or biopsy of the site to determine the organism(s) present, with subsequent appropriate antimicrobial therapy, can result in better HSCT outcomes.

3.3 Role of Radio-Labeled MIBG Imaging in Children with Neuroblastoma

^{123}I-MIBG is used for diagnostic purposes in patients with neuroblastoma. It is usually used as a whole-body scan, but can be used to focus on a particular area when combined with CT scanning. It is often usually used to demonstrate 2-dimensional images, (scintigraphy) but can be 3-dimensional when used in combination with SPECT scanning.

3.4 Ongoing Clinical Trials with MIBI

There are presently 97 experimental clinical trials (active and open) exploring the roles, uses, indications and toxicities of MIBG treatment (reference=https://www.clinicaltrials.gov/ct2/results/browse?term=MIBG&brwse=cond_cat). The studies are open around the world (58 in USA, 14 in Canada, 21 in Europe, 3 each in the Middle East, and the Pacific, 2 each in East Asia and South America, and 1 in North Asia). Of these studies, 48 are specifically for patients with neuroblastoma. Other MIBG studies are for patients with pheochromocytoma, autonomic dysfunction, myocardial sympathetic denervation, PVC ablation, cardiomyopathy, and others. Of the 48 studies presently registered at ClinicalTrials.Gov, 18 are open and accruing new patients with a known status. The trials have been divided into two categories (1) diagnostic use and (2) therapeutic use. This section covers the diagnostic use of the MIBI scanning.

3.5 Diagnostic Use of MIBI

The diagnostic uses for ^{123}I-MIBG imaging are; confirmation of diagnosis, localization for biopsy planning, prognostication of neuroblastoma when used as part of a scoring system, response criteria monitoring, presence or absence of minimal residual disease, and surveillance follow up for disease recurrence.

Scoring systems which use MIBG scanning are listed in Table 3.1. In each of the studies, MIBG scanning was evaluated for number of MIBG-avid sites as they related to outcome. In one study, Ady et al. [11] showed that mid-induction scores could predict overall response at the end of induction therapy. In another study, Suc et al. [12] showed that 4 or more sites on MIBG scans were associated with a higher risk for not achieving remission, while Matthay et al. [13] showed that three or more sites identified patients at higher risk for not responding to treatment. In one study, Katzenstein et al. [14] showed that MIBG scans correlated with outcome after stem cell transplantation.

In addition to scoring systems, ^{123}I-MIBG scanning can also be used to identify the type of metastatic spread, which may also relate to prognosis. MIBG scans present in different patterns: 'limited focal' and 'diffuse-metastatic'. The limited and focal pattern (median 2 body parts affected) was found in patients with MYCN amplification and correlates to better event-free and overall prognosis [15]. Whereas, extensive diffuse pattern (median 11 body parts affected) was found mainly in patients with single copy MYCN oncogene, and was associated with overall poorer prognosis [15].

A recent Cochrane Review [16] evaluated all of the published studies done through 2012 relating to either ^{123}I MIBG (SPECT) and/or ^{18}F-FDG (^{18}F-fludeoxyglucose) PET (CT) scanning as diagnostic studies in patients with neuroblastoma. The investigators identified 11 high quality studies involving 621

Table 3.1 MIBI scoring system

Stage of NB		Timing of MIBG scintigraphy	Result
1	Stage IV NB	MIBG scans at beginning, mid-course (6 weeks), end of neoadjuvant chemotherapy (12 weeks)	Mid-induction scores predict the overall response of metastases at the end of induction
2	Stage IV NB	MIBG scan at diagnosis	MIBG at diagnosis with four or more spots was associated with higher risk of failing to achieve remission
3	> 1 year and Stage IV NB	123I MIBG scans at diagnosis, after two and four cycles of induction therapy and before auto-SCT	Absolute and relative scores showed correlation with overall pre-transplantation response, bone marrow response and EFS
4	High risk NB	Post induction MIBG score in high risk NB patients treated with induction regimen, consolidated with 3 cycles of high dose therapy and stem cell rescue.	MIBG scores >/= 3 following induction identified NB patients likely to relapse

children with neuroblastoma under 18 years of age, who underwent either or both of these scanning procedures as adjuncts to their other diagnostic studies.

They found that the sensitivity of ^{123}I MIBG varied from 67 to 100% in patients with neuroblastoma. Thus, false negative findings occurred in as many as 33% of patients with neuroblastoma; this could result in under-estimating the severity and prognosis of patients and exposing them to inadequate treatment. Whether ^{18}F-FDG PET scans would identify disease in these ^{123}I-MIBG lesions was not addressed in any of the studies, and whether higher doses of the radioisotope would identify these lesions was also not addressed. Another potential limitation is lower ability to detect CNS metastases [17]. In contrast, there were exceedingly few false positive findings, which would have wrongly classified a patient as having metastatic disease, when they didn't and thereby subject the patient to excessive therapy.

^{123}I MIBG scanning may be inaccurate in the presence of interfering drugs including opioids, tricyclic antidepressants, sympathomimetics, anti-hypertensives, and some anti-psychotics. For children, these medications should be discontinued at least several days prior to the procedure, as possible. Alternatively, another type of scan may be preferred.

The side effects of MIBI scanning include; thyroid ablation, pancytopenia, thrombocytopenia, leukopenia and secondary hematologic malignancies [18, 19]. Non-hematologic toxicities include nausea, vomiting, seizures, allergic reactions, and increased risk of veno-occlusive disease in patients who subsequent undergo stem cell transplantation.

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Chapter 4
Clinical Applications of Stem Cell Transplant in Treating Non-Hematologic Conditions

Roopa Ram, Kedar Jambhekar, and Tarun Pandey

4.1 Introduction

Stem cells are undifferentiated cells, which are capable of dividing themselves even after long periods of inactivity, and have the potential to differentiate into several subtypes and become more specialized. The two properties of stem cells are their unique ability to divide infinitely and capacity to differentiate into several different specialized cells. Based on their commitment to become a particular type of cell, stem cells have been characterized into totipotent, pluripotent and multipotent cells (Table 4.1).

Stem cells can also be sub-classified into embryonic, adult tissue stem cells and induced pluripotent cells based on their source of origin (Table 4.2). Furthermore, based on the tissues in which they occur and the cell lines which they can form, adult stem cells are sub classified into several different types such as hematopoietic stem cells, mesenchymal stem cells, neural stem cells, epithelial stem cells and skin stem cells. Hematopoietic stem cells found in bone marrow can differentiate into red blood cells, white blood cells, platelets. Mesenchymal stem cells found in various tissues including the bone marrow, can differentiate into cells that form bone, cartilage, fat and stromal cells (Fig. 4.1).

Due to their ability to infinitely divide and form new cells as well as their unique power to differentiate and specialize into several subtypes of tissues, stem cells have evoked significant research interest in the past few decades. Wide range of clinical applications for use of stem cells have been found, the most promising ones being in the field of hematology where hematopoietic stem cell therapy (HSCT) has been successfully used in treating both malignant causes (lymphoma, leukemia, myeloma) and non-malignant hematologic causes (chronic anemia, polycythemia etc.).

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Table 4.1 Types of stem cells

Subtype of stem cell	Capability to differentiate
Totipotent	Capable of forming an entire organism (sperm/ovum)
Pluripotent	Capable of forming several different tissue types
Multipotent	More differentiated, Capable of forming limited types of cells within specialized tissues

Table 4.2 Classification of stem cells

Subtype of stem cell	Origin
Embryonic stem cell	Derived from embryos
Adult stem cell	Found in adult tissues (bone marrow, skin, gut, neural tissue etc.)
Induced pluripotent stem cell	Reprogrammed adult stem cells to become less differentiated and behave like embryonic cells

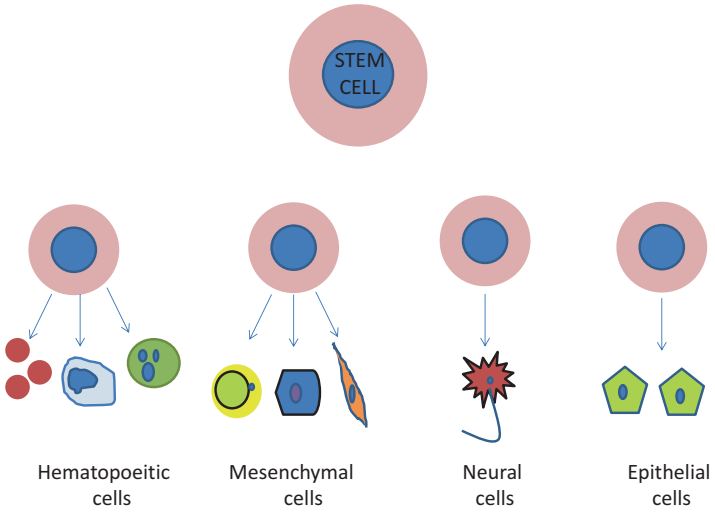

Fig. 4.1 Classification of stem cells

However, both hematopoietic stem cell therapy (HSCT) and mesenchymal stem cell therapy (MSCT) have clinical implications in various non-hematologic conditions including multi organ degenerative disorders, metabolic and endocrine disorders, rheumatologic and autoimmune diseases. In this article, we will review the non-hematologic applications of stem cell therapy, focusing particularly on the use of mesenchymal cells in multi system disorders. We will also briefly discuss the role of imaging in some of these evolving clinical applications of mesenchymal stem cells.

4.2 Mesenchymal Stem Cells: Unique Attributes

As stated above, mesenchymal stem cells are pluripotent cells found in various organs including bone marrow, adipocytes, placenta, amniotic fluid and umbilical cord blood. Certain specific criteria as defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy are required for stem cells from these various sources to be considered standard stem cells and equivalent to each other [1]. These include spindle cell morphology, adherence to plastic, ability to differentiate into adipocytic, chondrogenic and osteogenic cell lines and expression of certain specific cell markers ($CD73^+$ $CD90^+$ $CD105^+$ $CD34^-$ $CD45^-CD11b^-$ $CD14^-$ $CD19^-CD79a^-$ $HLA\text{-}DR^-$).

Mesenchymal stem cells have certain unique attributes that make them suitable for usage in multiple organ systems. These are summarized in Table 4.3.

In addition to their ability to divide infinitely and differentiate into several mesodermal cell lines such as adipocytes, chondrocytes and osteocytes, mesenchymal stem cells in several in vitro models have also known to have the capacity to differentiate into non-mesodermal cell lines and thus have found applications in organ systems such as liver, brain and heart. Although several in vivo models of injury have shown engraftment and differentiation of mesenchymal cells into various other cell lines such as hepatocytes, neural cells and cardiomyocytes, it is unknown if these effects are solely due to trans differentiation or due to other effects such as paracrine effects as well [2, 3].

Mesenchymal cells also exert immuno suppressive and anti inflammatory properties, predominantly by suppressing T lymphocyte and natural killer cell proliferation, B cell function and cytokine production. Several products such as transforming growth factor-beta, hepatocyte growth factor assist in the anti inflammatory properties of these cells [4].

Mesenchymal cells are also known to selectively migrate to sites of active inflammation in the body, mainly due to interaction with specific receptors such as chemokine receptor 4, stromal cell derived factor receptor, vascular endothelial growth factor receptor and platelet derived growth factor receptor [5]. Injured tissues express higher proportion of these receptors and thus facilitate homing of mesenchymal cells selectively.

Due to the above mentioned attributes and the ease with which they can be isolated as well as their low immunogenicity and high tolerance in tissues, mesenchymal stem

Table 4.3 Unique attributes of stem cells

1. Ability to differentiate into both mesodermal and non mesodermal lineages
2. Immune modulation
3. Migratory capacity and homing in
4. Paracrine effects
5. Easy isolation and ex vivo expansion

cells have been extensively studied in several human clinical trials involving multiple organ systems. Some of the common clinical indications where there has been success in human models are described in the following sections and are summarized in the following Table 4.4.

4.3 Central Nervous System

4.3.1 Brain and Spinal Cord Diseases

Stem cells have found several applications in treating common neurological conditions. Some of the diseases where stem cells have been studied are summarized in Table 4.5 and a short summary is included below.

4.3.1.1 Amyotrophic Lateral Sclerosis (ALS)

ALS is a chronic neurodegenerative disorder characterized by progressive motor neuron dysfunction and paralysis. While several treatment options have been described for treatment, due to their immune-modulatory function mesenchymal stem cells (MSCs) have been extensively studied in halting progression of disease [6].

Table 4.4 Potential non-hematopoietic applications of stem cells

Central nervous system	Alzheimer's, Multiple sclerosis, Amyotrophic lateral sclerosis, Stroke, Spinal cord injury, Autism, hearing loss, cerebral palsy
Musculoskeletal system	Osteoarthritis, Rheumatoid arthritis, Degenerative disc disease, Osteogenesis imperfecta, Osteopetrosis, Solid tumors (sarcoma)
Gastrointestinal system	Liver disease, Inflammatory bowel disease
Cardiovascular system	Acute myocardial infarction, Congestive heart failure
Pulmonary	Chronic obstructive pulmonary disease, Bronchopulmonary dysplasia
Endocrine disorders	Type 1 Diabetes
Autoimmune disorders	Systemic lupus erythematosus, Systemic sclerosis
Metabolic disorders	Metachromatic leukodystrophy, Hurler syndrome

Table 4.5 Diseases of the brain and spinal cord where stem cells are used

Amyotrophic lateral sclerosis (ALS)
Multiple sclerosis (MS)
Neuromyelitis optica (NMO)
Alzheimer's disease (AD)
Spinal cord injury (SCI)
Stroke

Multiple ongoing clinical trials where MSCs are being used for their trophic and immune-modulatory effects have also been described [7].

Various routes of administration of mesenchymal stem cells have been described in the literature including intravenous and intrathecal routes with no reported adverse effects. Some of the recent data has also suggested that use of mesenchymal stem cell therapy may halt progression of bulbar symptoms and respiratory paralysis in ALS patients [8].

4.3.1.2 Multiple Sclerosis (MS)

MS is a chronic neurodegenerative disease characterized by relapsing and remitting episodes of neurological symptoms. The relapsing-remitting form of MS is the most common subtype and occurs in young patients and is characterized by T-cell mediated immune response, which triggers a cascade of events, the end result of which is demyelination and axonal damage. Due to their immune-modulatory and immune-suppressive effects, hematopoietic stem cell therapy has been well studied in the treatment of multiple sclerosis with best results seen in patients with active disease of shorter duration [9].

The outcomes of treatment are also known to be partly dependent on the pre transplant conditioning regimens with long-term progression-free survival being better in patients who received intermediate-intensity regimens conditioning versus high-intensity regimens [10].

In addition to hematopoietic stem cells, neural progenitor cells of mesenchymal origin have also been found to have therapeutic effects in both stages of multiple sclerosis in animal models due to their anti-inflammatory effect in the acute stage and neuro regenerative properties in the chronic stage [11].

Several reports of neuromyelitis optica, another related chronic demyelinating disease affecting the optic nerve and spinal cord being treated with umbilical cord derived mesenchymal stem cells have also been reported [12] with improvement in clinical course as well as resolution of active lesions as detected on MRI [13].

4.3.1.3 Alzheimer's Disease

Alzheimer's disease is a slowly progressive neurological disorder characterized by loss of memory and cognitive function. Although multiple factors such as accumulation of abnormal proteins such as amyloid beta and ApoE4 are involved in the pathogenesis of Alzheimer's disease, the final common pathway is loss of cholinergic neurons and synaptic connections. This predominantly affects the basal ganglia, hippocampus and neocortical association areas of the brain. Therapies directed at treating Alzheimer's disease have focused on replacing and regenerating the impaired cholinergic neurons. Neural stem cells, due to their ability to replicate and differentiate into several different cell types such as neurons, astrocytes and oligodendrocytes at transplantation sites have been studied in management of Alzheimer's

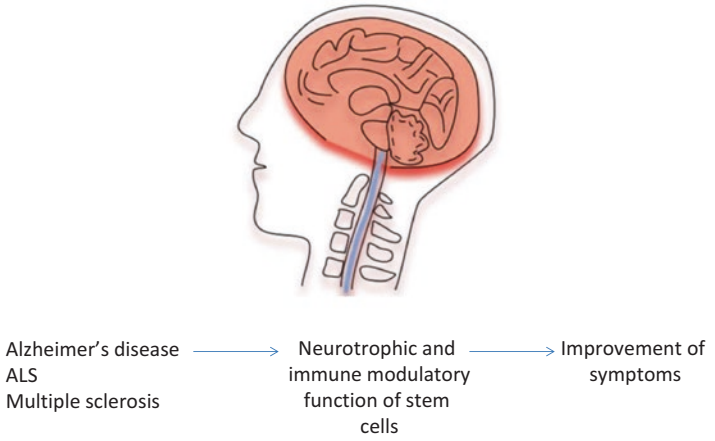

Fig. 4.2 Role of stem cells in brain disorders (*arrow*)

disease in a few animal model studies. Improvement in learning, memory and increase in cholinergic neuron number have been reported in animal models [14].

Due to their ability to migrate to areas of brain where there is cell damage, neural stem cells have also been used as vehicles to deliver neurotrophic factors such as glial cell derived neurotrophic factor as well serve as carriers of genes that produce neurotrophic factors (Fig. 4.2) [15].

A study, in which umbilical cord blood-derived mesenchymal stem cells co-culture reduced the hippocampal apoptosis induced by amyloid-β peptide treatment, was published by a group in South Korea. Moreover, Alzheimer's disease in a mouse model treated with umbilical cord-derived stem cells demonstrated cognitive rescue with restoration of learning/memory function [16].

4.3.1.4 Stroke

The role of stem cell therapy in ischemic stroke patients lies in restoring function and reducing damage to the brain cells. Due to their ability to cross blood brain barrier and preferentially home in damaged areas of the brain, mesenchymal stem cells have been shown to promote cellular regeneration by increasing neovascularity, expressing trophic factors that promote function of neural progenitor cells, as well as reduce apoptosis [17]. Studies performed on stroke patients injected with intravenous mesenchymal stem cells have shown good safety profiles and clinical improvement in neurologic deficits as well as reduction in atrophy within peri-infarct areas as seen on follow up MRI at 12 months [18].

4.3.1.5 Spinal Cord Injury (SCI)

Spinal cord injury is a chronic debilitating condition that results in loss of sensory-motor and other neurological functions such as bowel and bladder control. A wide variety of medical and surgical treatments have been tried to aim at restoring the morphological structure of the spinal cord and to restore its function. Due to their ability to rapidly proliferate and differentiate into several different cell lines, stem cell therapy has been tried in altering the course of symptoms in patients with spinal cord injury. Several parameters such as clinical neurologic improvement, motor and sensory evoked potentials, bladder urodynamic testing have been studied following stem cell therapy in SCI patients and have shown promising results [19].

4.3.2 Musculoskeletal System

4.3.2.1 Osteoarthritis

Osteoarthritis is a progressive degenerative disorder, often affecting large weight bearing joints such as hips and knees. Prior trauma or altered mechanics due to various factors may accelerate this process. Several experimental treatments to reduce cartilage damage and slow progression of cartilage loss including micro fracture and autologous chondrocyte implantation have been described [20]. However, due to their unique ability to rapidly proliferate and differentiate into chondrocytes, mesenchymal stem cell implants have been studied for their potential role in managing osteoarthritis and may have a role in halting progression of osteoarthritis [21].

In addition, mesenchymal stem cells produce several cytokines and growth factors, the paracrine effect of which helps in tissue regeneration and angiogenesis [22].

Recent studies in patients treated with autologous mesenchymal stem cells for cartilage defects have shown promising results with improved arthroscopic and histologic grading when compared to un-treated patients [23–25]. Largest of these was a study of 56 patients who underwent intra articular injection of a combination of autologous mesenchymal stem cells and hyaluronic acid versus hyaluronic acid alone 3 weeks following high tibial osteotomy and microfracture for osteoarthritis. Improved clinical outcome as well as MRI appearance was observed in the treated group of patients as measured using IKDC (International Knee Documentation Committee) score at 6 months, 1 and 2 years and MOCART (Magnetic Resonance Observation of Cartilage Repair Tissue) scores at 1 year respectively [25].

4.3.2.2 Rheumatoid Arthritis

Rheumatoid arthritis is a chronic synovial inflammatory arthritis mediated by activated T lymphocytes, eventually resulting in cartilage loss and joint destruction. Due to their ability to suppress the proliferation of activated T lymphocytes,

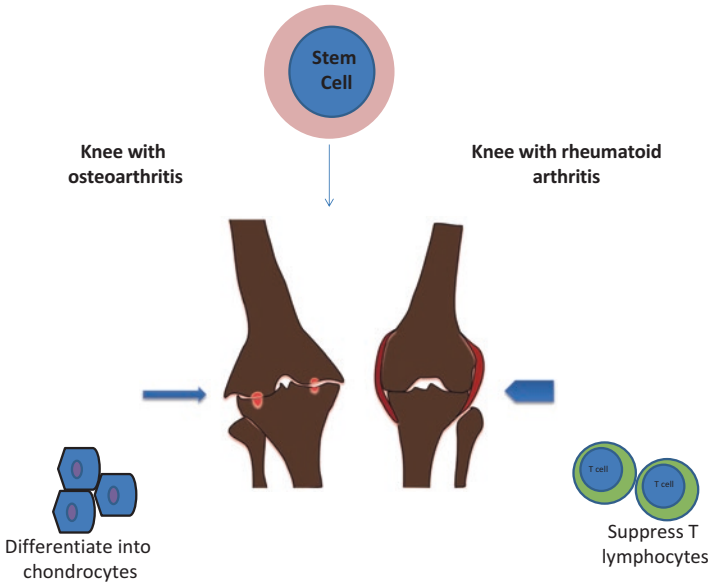

Fig. 4.3 Role of stem cells in osteoarthritis (*arrow*) and rheumatoid arthritis (*arrowhead*)

mesenchymal stem cells including those derived from synovial origin are useful in rheumatoid arthritis [26].

Newer clinical trials in a group of 172 patients treated with combination intravenous injection of umbilical cord derived mesenchymal stem cells and disease modifying anti-rheumatoid drugs (DMARDs) have shown good safety profiles, decrease in serological inflammatory markers such as tumor necrosis factor alpha and interleukin-6 and improvement in clinical course of disease compared to patients who were treated with DMARDs alone (Fig. 4.3) [27].

4.3.2.3 Osteogenesis Imperfecta (OI)

Osteogenesis imperfecta is an inherited disorder resulting in fragile, deformed bones and growth retardation and results from a mutation in genes encoding one of the alpha chains of collagen type 1, which is the main scaffolding for bone formation. Stem cell therapy due to it's regenerative potential into osteoprogenitor cells such as osteoblasts has been studied in small group of patients with OI [28]. Patients who were pre treated with bone marrow transplantation followed by mesenchymal stem cells injection showed greater engraftment of cells in defective bone, increase in growth rate and reduced number of fractures, thus validating the role of mesenchymal stem cells in extending the benefits of bone marrow transplant in OI patients.

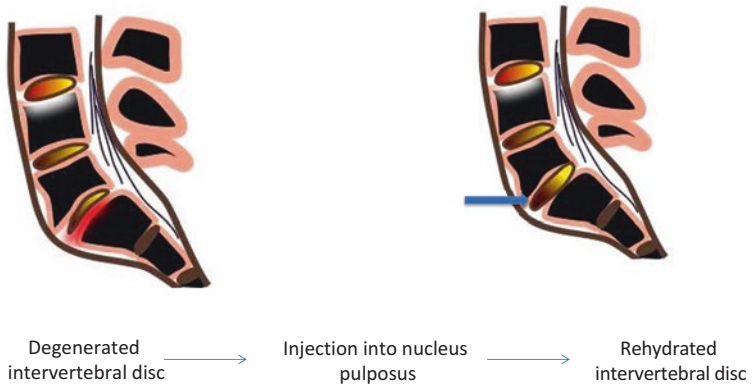

Fig. 4.4 Role of stem cells in degenerative disc disease with improved hydration after treatment (*arrow*)

4.3.2.4 Degenerative Disc Disease

Intervertebral discs are fibro cartilaginous structures that stabilize the spine and allow motion between vertebral bodies. Discs are composed of a central cellular and gelatinous nucleus pulposus and a tougher outer collagen containing annulus fibrosus. Age related disc degeneration mediated by increased expression of enzymes that degrade the extracellular matrix around the disc, eventually result in loss of disc hydration and disc height. This wear of the disc result in association with facet joint hypertrophy can result in spinal canal stenosis and clinically present with back pain.

Due to their ability to stimulate endogenous intervertebral disc cells and promote formation of extracellular matrix and thus restore the structure of the degenerating disc, stem cells have been studied in several clinical trials. One of these studies performed in ten patients 1 year after local injection of mesenchymal stem cells into the nucleus pulposus showed both clinical (improved back pain and disability) as well as radiological improvement (improvement in water content of the disc as seen on T2 weighted images) [29]. Beneficial effects using MRI have been assessed in several other similar studies with improvement seen in parameters such as increased T2 signal and height of the disc [30] (Fig. 4.4).

4.4 Gastrointestinal System

4.4.1 Cirrhosis

Cirrhosis of the liver is the end result of several pathologic processes such as chronic viral hepatitis, long term alcohol use, fatty liver, drugs and several other causes. Cirrhosis carries high morbidity and mortality with liver transplantation being the only definitive treatment. However, owing to shortage of donor livers world wide,

Table 4.6 Mechanism of action of stem cells in the liver

Differentiation into hepatocyte like cells
Secretion of trophic factors (which regulate hepatic stellate cell activity and inhibit fibrosis as well as promote regeneration of damaged liver)
Immune modulation

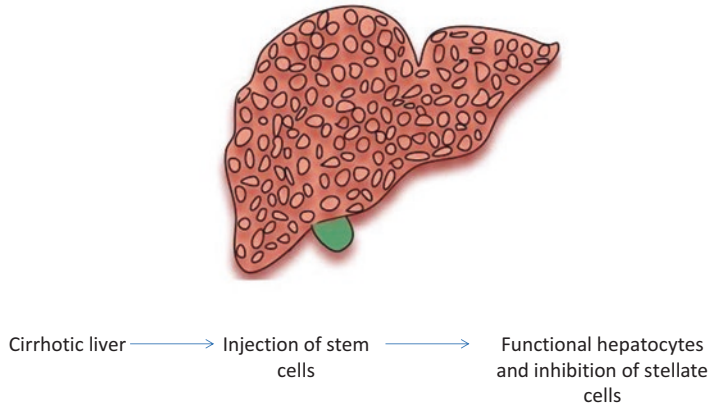

Fig. 4.5 Role of stem cells in cirrhosis

significant research into other therapeutic options, which can alter the course of disease, are being performed and multiple clinical trials have been reported.

Stem cell therapy and their mode of action have been studied in the management of cirrhosis (Table 4.6, Fig. 4.5). In addition to their ability to differentiate into functional hepatocyte like cells, mesenchymal stem cells play a major role by secreting several trophic factors. Some of these trophic factors modulate the activity of hepatic stellate cells that are important cells that promote fibrosis in the liver. Thus, trophic factors derived from mesenchymal stem cells act by inhibiting the proliferation of stellate cells and promoting their apoptosis. Several routes of administration of stem cells including administration through peripheral veins, portal vein and hepatic artery have been described [31].

Meta analysis of several clinical trials have proven that treatment with bone marrow derived mesenchymal stem cells showed improvement in several parameters of liver function such as serum albumin, prothrombin concentration, total bilirubin and MELD (Model for end stage liver disease) score [32].

4.4.2 Inflammatory Bowel Disease

Inflammatory bowel disease, which includes Crohn's disease and ulcerative colitis, is a complex immune mediated chronic gastro intestinal inflammatory disease that occurs in genetically predisposed individuals as a result of interaction between

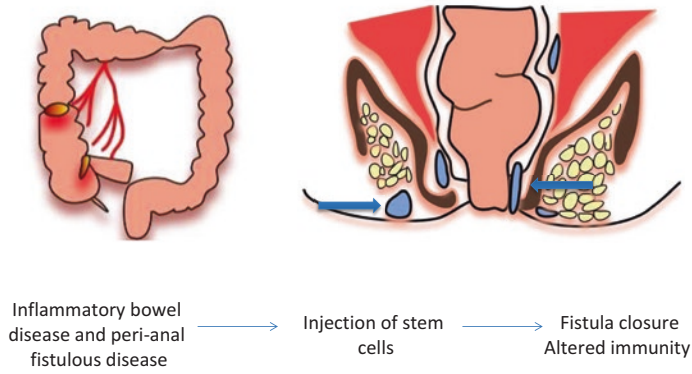

Fig. 4.6 Role of stem cells in inflammatory bowel disease and in treatment of peri-anal fistula (*arrows*)

environmental and dietary factors. Antigens derived from dietary and environmental cause stimulate excessive inflammatory response within the bowel, which results in trans-mural inflammation and complications such as fistula formation and bowel wall fibrosis in the long term.

Several forms of stem cell therapy such as hematopoietic and mesenchymal stem cell therapy have been tried in treating Crohn's disease, primarily due to the immune modulatory effects of these cells. Therapies such as autologous and allogenic stem cell therapy are aimed at altering the immune response generated by the body to various antigens rather than targeting the antigens themselves. Proposed mechanisms of action for autologous and allogenic stem cell therapy include resetting the patient's immune system by suppression of T lymphocytes and providing a new immune system respectively.

MSC are used in management of Crohn's disease due to their ability to inhibit fibrosis and inflammation as well as promote tissue healing. Both systemic therapy through intravenous and intra arterial routes and localized therapy in fistulizing Crohn's disease have been described (Fig. 4.6) [33]. While the data on systemic mesenchymal stem cells is limited, several ongoing phase 3 clinical trials have reported no adverse side effects [34].

The data for local therapy is more promising with healing of fistulae as well as complete long-term fistula closure reported in patients with perianal disease who were injected with adipocyte derived mesenchymal stem cells [35]. The most recent of these data is from a series of 33 patients who were studied at 1 year following local peri anal injection and showed long-term fistula closure in 88% cases [36].

4.5 Pulmonary Diseases

Due to their ability to regenerate pneumocytes, remodel the extra cellular matrix and immune suppressive effects, mesenchymal stem cells have been studied in treating several pulmonary conditions. Unlike in most organs where more targeted routes of administration have to be performed to circumvent the first pass effect that occurs in the pulmonary circulation, simple intravenous infusion can be directly used in treating pulmonary conditions.

4.5.1 Chronic Obstructive Pulmonary Disease (COPD)

COPD is a chronic inflammatory process affecting the airways, which results in irreversible alveolar damage and progressive decline of pulmonary function. Due to their immune-modulatory effects and ability to regenerate type 1 and type 2 pneumocytes, mesenchymal stem cells have been tested in several clinical trials in patients with COPD. Although some of the preliminary larger studies did not show significant change in pulmonary function tests or change in quality of life indicators, they showed good safety profiles and reduction in inflammatory markers such as C-reactive protein at 2 year follow up [37].

Recent studies however with smaller sample size have shown improvement in pulmonary function tests as well as improvement in quality of life, thus making this a promising treatment option for COPD patients [38].

Other pulmonary conditions where stem cells have been studied include idiopathic pulmonary fibrosis and pulmonary hypertension. Endobronchially administered adipose derived mesenchymal stem cells studied in Idiopathic pulmonary fibrosis (IPF) in phase 1 clinical trials have shown good safety, although their clinical efficacy still needs to be determined [39]. Similarly, mesenchymal stem cells in animal models have been studied in treating pulmonary arterial hypertension as well [40]. Further studies are needed to extrapolate this data to human subjects.

4.6 Cardiovascular Diseases

Due to their ability to differentiate into cardiomyocytes and endothelial cells, mesenchymal stem cells have been shown to be useful in regenerating diseased myocardium as well in promoting angiogenesis (Fig. 4.7). Several routes of administration of these cells including intra myocardial and intracoronary have been described in the literature.

In a study published in 2004, 69 patients with acute myocardial infarction who were treated with intra coronary mesenchymal stem cells within 12 h after presenting showed significant decrease in ischemic extent at 3 months and improvement in

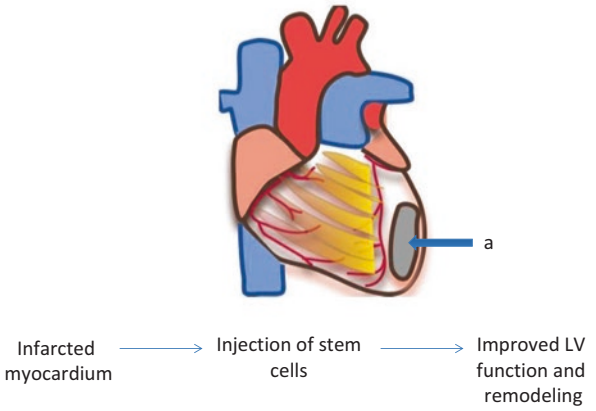

Fig. 4.7 Role of stem cells in myocardial infarction (*arrow*)

ejection fraction at 6 months, thus lending support to the theory that mesenchymal stem cells promote remodeling of the left ventricle [41].

In a more recent study, 53 patients with first episode of myocardial infarction who were treated with intravenous mesenchymal stem cells vs placebo showed improved outcomes related to cardiac arrhythmias, pulmonary function, left ventricular function and symptomatic global assessment compared to placebo treated patients. Significant improvement in left ventricular ejection fraction as quantified by cardiac MRI was seen in the treated group and was maintained at 12 month follow up [42].

4.7 Endocrine Diseases

4.7.1 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus is a chronic debilitating condition triggered by autoimmune mediated destruction of insulin producing pancreatic beta cells by activated T lymphocytes. In addition to life long insulin requirement, islet cell transplantation is one of the more definitive treatment options that has been tried. One of the first Islet cell transplantation techniques in six insulin dependent diabetic patients using transhepatic intra portal infusion was described by Neeman et al. using six patients, with improved glycemic control and lesser need for insulin following islet cell transplantation. Since then several studies have also described other routes and techniques to perform Islet cell transplantation. However, the limited supply of islet cells and adverse effects of life long immunosuppression have necessitated the use of safer alternatives such as use of stem cells [43].

Mesenchymal stem cells due to their immune-modulatory properties may have a role in inhibiting the function of T-lymphocytes which cause destruction of newly formed beta cells. Also due to their trophic and angiogenic properties, mesenchymal

stem cells have also shown to limit the damage to existing beta cells and stimulate their growth and differentiation [44].

Several clinical trials have been performed to test the safety and efficacy of mesenchymal stem cells, with improved glycemic control and reduction in C peptide levels reported in a series of 29 patients with newly diagnosed type 1 diabetes treated with Wharton's jelly derived mesenchymal stem cells in a 2 year follow up [45].

In addition to their role in type 1 diabetes, patients with type 2 diabetes have also shown to benefit from mesenchymal stem cell therapy. Studies performed in patients with type 2 diabetes and co-existing chronic myocardial ischemia have shown reduction in size of infarction and improvement in cardiac function following trans endocardial injection of mesenchymal stem cells [46].

4.8 Autoimmune Diseases

Systemic lupus erythematosus (SLE) and systemic sclerosis (SS) are examples of autoimmune diseases, where a patient's immune system attacks and damages its own body.

4.8.1 Systemic Lupus Erythematosus

SLE is characterized by auto-immune antibodies to RNA binding proteins and double stranded DNA, necessitating life-long immune-suppression. Due to their immune-modulatory properties and ability to inhibit T cell proliferation, mesenchymal stem cells have been the subject matter of clinical trials.

Small group studies consisting of 16 patients treated with mesenchymal stem cells, with upto 2 years of follow up have shown improvement in renal function and serologic markers of lupus such as serum antinuclear antibody, serum anti double stranded DNA, complement C2 levels, without significant adverse effects [47].

Additional smaller but newer study with four patients has showed benefits of using mesenchymal stem cells in treating diffuse alveolar hemorrhage with improvement in parameters such as improved oxygen saturation to lungs, improved hemoglobin levels and platelet count compared to pre-treatment levels [48].

Studies have also shown beneficial role of mesenchymal stem cells in improving disease activity and blood cell counts in patients when used in a series of 35 patients with lupus and refractory pancytopenia [49].

4.8.2 Systemic Sclerosis

Systemic sclerosis is a chronic progressive connective tissue disorder characterized by excessive collagen deposition in skin and internal organs. Diffuse skin involvement in the form of skin thickening and digital ulcers is very common. Systemic

complications such as interstitial lung disease and renal failure can complicate the disease in the long term. Abnormal T cell activation, autoantibody production (anti SCL-70) and cytokine production have been incriminated in the pathogenesis of the disease. Extensive studies using hematopoietic stem cells have been studied in the management of systemic sclerosis and include the ASSIST, ASTIS and SCOT trials [50]. While newer small group studies have reported improvement in digital ulcers and improved blood supply to the digits following intravenous mesenchymal stem cell infusion, with no significant adverse side effects; larger trials to validate such findings are pending [51, 52].

4.8.3 Inflammatory Myopathies

Dermatomyositis and polymyositis are inflammatory myopathies which are characterized by proximal muscle weakness and elevated muscle enzymes such as creatine kinase. These are thought to be mediated by complement activation system and cytotoxic CD8+ T cells respectively, which induce myonecrosis [50].

Small studies using mesenchymal stem cells in patients refractory to therapy for these conditions have reported improvement in muscle strength, reduction in creatine kinase levels as well as improvement in interstitial lung disease associated with these inflammatory myopathies [53].

4.9 Inborn Errors of Metabolism

4.9.1 Metachromatic Leukodystrophy and Hurler Syndrome: (MLD)

MLD is an inherited disorder characterized by lack of enzyme arylsulfatase-A that affects the white matter, resulting in progressive demyelination of both central and peripheral nervous systems. No definitive cure exists for this disorder that results in progressive loss of cognitive and motor function.

Several studies have reported the efficacy of hematopoietic stem cell transplantation in stabilizing the course of the disease with some case reports showing long-term improvement in MRI appearance of white matter lesions as well as spectroscopy findings in patients followed for over 2 years after transplantation. However due to limited availability of matched donors, limited entry of hematopoietic stem into the brain and greater side effects, alternate cell based therapies such as use of mesenchymal cells have been studied [54]. Due to their ability to differentiate into neuronal cells, ability to migrate in the brain, including the “homing” phenomenon where they selectively accumulate in damaged/inflamed areas of the brain, mesenchymal cells have been studied in treating MLD. In a small study of patients concomitantly treated

with hematopoietic stem cell therapy and mesenchymal stem cell therapy, significant improvement in nerve conduction velocities was noted, suggesting that mesenchymal stem cells facilitate the action of hematopoietic stem cells and may differentiate into Schwann cells or produce the enzyme arylsulfatase themselves [55].

4.10 Conclusion

Mesenchymal stem cells have the unique ability to differentiate into various cell lines, selectively home in injured/inflamed tissues, promote growth of endogenous cells by producing trophic factors, possess anti inflammatory and immune modulatory effects and; have found various applications in multiple organ systems. Several animal models and ongoing human clinical trials have all mostly established the safety of mesenchymal stem cells, although the long term efficacy remains to be well established and thus far has only been studied in small groups of human subjects. Larger multi center randomized controlled studies are needed to validate the results of smaller studies. Although imaging modalities such as MRI have been described in evaluating response to musculoskeletal and neurological applications of mesenchymal stem cells, further studies to validate the preliminary findings are pending.

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Part II
Applications in Stem Cell and Cell Based Therapies

Chapter 5
Stem Cell Transplantation for Multiple Myeloma

Sharmilan Thanendrarajan and Tarun K. Garg

5.1 Multiple Myeloma

Multiple myeloma (MM) is a malignant blood disorder which is characterized by accumulation of abnormal, clonal plasma cells (myeloma cells) in the bone marrow with production of complete and/or partial (light chain) monoclonal immunoglobulin protein which can be detected in serum and/or urine [1]. The expansion of plasma cells in the bone marrow leads to impairment of hematopoiesis with leucopenia, anemia and thrombocytopenia, resulting in clinical symptoms consisting of recurrent infections, persisting fatigue and bleedings [2]. Further major characteristics of this fatal disease are bone lesions with pathological fractures and/or cord compression, renal insufficiency (related to cast nephropathy) and hypercalcemia [2]. Hypercalcemia (C), renal insufficiency (R), anemia (A) and bone lesions (B) are summarized as CRAB-criteria and stand for myeloma defining events [3]. Figure 5.1. provides an overview and summary of the diagnostic criteria for MM according to the International Myeloma Working Group (IMWG). MM is the second most common hematological malignancy and contributes to 1% of all malignant tumors [2, 4]. The median age at diagnosis is 70 years, and more than 60% of the newly diagnosed patients are elder than 65 years [2].

MM arises from asymptomatic premalignant expansion of monoclonal plasma cells in the bone marrow that are derived from post–germinal-center B cells. Several additional microenvironmental and genetic changes lead to the transformation of these abnormal plasma cells into a malignant neoplasm. The first stage in this process is defined as monoclonal gammopathy of undetermined significance (MGUS) that progresses to smoldering myeloma and finally to symptomatic MM which required systemic treatment [5]. The risk of progression from MGUS to MM

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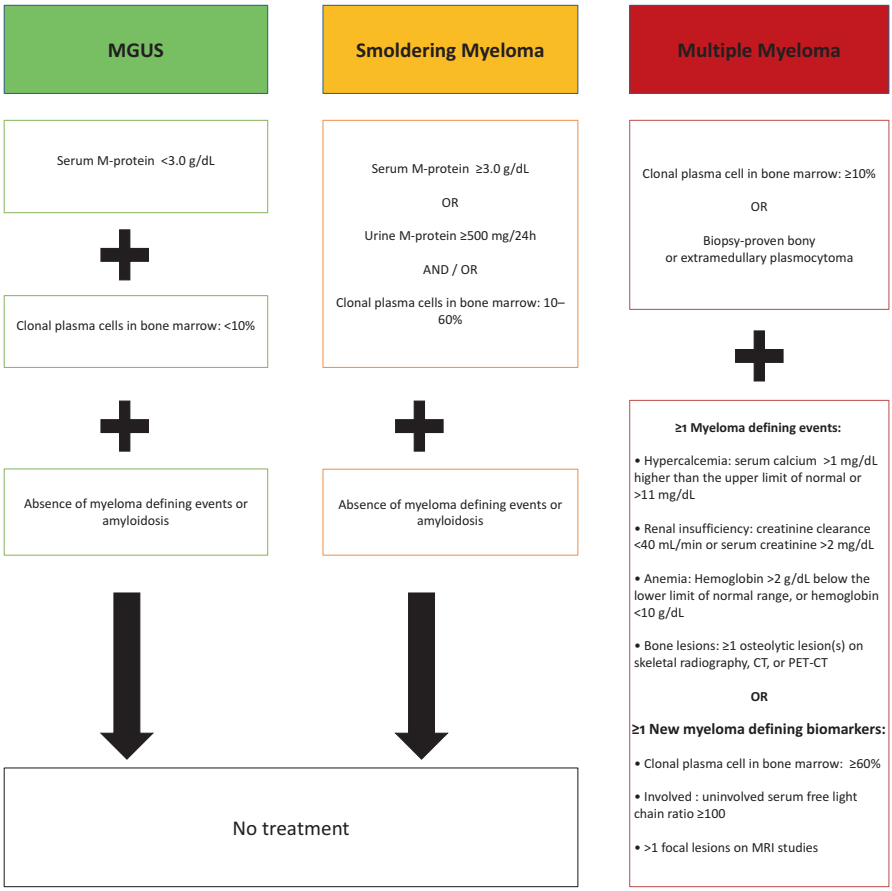

Fig. 5.1. Diagnostic criteria for multiple myeloma, smoldering myeloma and monoclonal gammopathy of undetermined significance

amounts to 0.5–1% per year [5]. MM is a very heterogeneous malignant disorder with refractory and relapsing course of disease that can be classified as low and high-risk [6]. High-risk MM is characterized by presence of extramedullary disease, larger than 20% plasma cell in peripheral blood (plasma cell leukemia), unfavorable genetic alterations (deletion 17p, deletion 1p, gain 1q21, t(14;20), t(14;16)), and poor clinical outcome and overall survival (OS) [6–8].

The introduction of high-dose (HD) chemotherapy and autologous bone marrow transplantation (ABMT) 30 years ago has revolutionized the treatment of patients with MM [9]. It is general consensus among myeloma experts that HD-chemotherapy and autologous stem cell transplantation (ASCT) is the backbone for treatment in newly diagnosed MM patients [10]. However, in the era of novel, potent anti-myeloma agents the role of ASCT needs to be redefined. The treatment landscape has dramatically changed in recent years with the introduction of highly effective

drugs for newly diagnosed and refractory/relapsing MM patients, such as proteasome inhibitors (bortezomib, carfilzomib, ixazomib), immunomodulatory drugs (IMiDs) (thalidomide, lenalidomide, pomalidomide), histone deacetylase (HDAC) inhibitors (panobinostat) and most recently monoclonal antibodies (daratumumab, elotuzumab) [7, 8, 11–19].

Kumar et al. have demonstrated that the application of novel anti-myeloma agents has significantly improved the OS rates in newly diagnosed (44.8 vs 29.9 months; $p < 0.001$) and relapsed (23.9 versus 11.8 months; $p < 0.001$) MM patients [4]. Novel agents including proteasome inhibitors and IMiDs are routinely used as part of the induction therapy before autologous transplantation which has resulted in substantial improvement in the depth of response achieved before transplant [20]. It is general practice to use a combination of a least three different drugs for induction therapy prior to ASCT in order to achieve a high response and durable complete remission (CR) rate [20]. In addition, the application of consolidation and maintenance therapy in the post-ASCT setting is an increasingly attractive treatment concept for patient with MM. Several studies demonstrate that consolidation and maintenance post-ASCT can further reduce tumor burden and improve clinical outcome [21].

Cure in MM is not anymore an unachievable goal [22]. A certain number of myeloma patients, in particular low-risk myeloma patients, can achieve long-term unmaintained, stringent-defined CR with OS rates of over more than 15 years [22].

5.2 HD-Chemotherapy and ASCT

5.2.1 Historic Background

In 1958, Blokhin et al. introduced melphalan as alkylating agent for treatment of six patients with MM leading to considerable reduction in tumor size in half of the patients [23]. In 1968 Alexanian et al. demonstrated that low-dose (LD) melphalan (0.2 mg/kg/day or 0.7–1.3 mg/kg over 4 days, every 6 weeks) is effective in patient with MM: from 82 myeloma patients 49% (n = 40) had an objective response rate with improvement of clinical status and outcome [24]. The application of LD-melphalan in combination with prednisone further improved OS in myeloma patients compared to patient who only received LD-melphalan [25].

The experience with LD-melphalan has led to the introduction of dose-escalated HD-melphalan in patients with MM in order to overcome drug resistance. In 1983 McElwain and Powles [26] administered HD-melphalan (100–140 mg/m^2) in eight patients with MM and one patient with plasma cell leukemia. All of the patients responded to the treatment and three out of the previously untreated patients (n = 5) even achieved CR. Despite these impressive results the clinical outcome was impaired by the unacceptably high lethality rate of 20% and long aplasia phase with neutropenia of 5–6 weeks [27]. The phase of aplasia was even 2–3 weeks longer in myeloma patients who had prior treatment [27, 28].

In order to achieve a quicker hematopoietic recovery and prevent infectious and bleeding complications after HD-chemotherapy, ABMT was introduced in 1986. Sixteen refractory myeloma patients were treated with 80–100 mg/m^2 melphalan solely, and seven patients with 140 mg/m^2 melphalan with ABMT. A reduction of the tumor mass by more than 75% was noted in 14 patients, including four who died of bone marrow aplasia. In six out of seven patients who received HD-melphalan with ABMT serious infections were prevented [9]. In 1987 Barlogie et al. applied HD-melphalan (140 mg/m^2) with total body irradiation (TBI) supported by ABMT in seven patients with advanced MM who were refractory to VAD (vincristine, adriamycin, dexamethasone). A very rapid response with larger than 90% reduction of tumor mass was accomplished in six patients with a median remission duration of 15 months, and five patients remained alive and well without further cytotoxic treatment for a median of more than 9 month [29].

Later on in 1990 the concept of Total Therapy was established by Bart Barlogie applying two ASCT (tandem) successively as backbone treatment for MM embedded in a treatment regimen consisting of induction (prior to ASCT), consolidation and maintenance therapy (after ASCT) [30]. Until now, tens of thousands of myeloma patients have been successfully treated with HD-chemotherapy and ASCT worldwide.

5.2.2 Chemotherapy Versus ASCT

It is general agreement among myeloma experts that HD-chemotherapy and ASCT is recognized as effective standard consolidative treatment in patients with MM. In 1996 the Intergroupe Francais du Myeloma (IFM) research group presented the first randomized study in 200 newly diagnosed myeloma patients receiving either conventional chemotherapy (CC) or HD-chemotherapy and ASCT as consolidation treatment. The response rate in the CC group was 57%, compared to 81% in the ASCT group with CR and very good partial remission (VGPR) rates of 5% and 9% only, compared and 22% and 16% ($p < 0.001$), respectively. The probability of event-free survival (EFS) for 5 years was 10% in CC, and 28% in ASCT-patients ($p = 0.01$). The estimated 5-year OS rate was 12% in the CC group, and 52% in the ASCT group ($p = 0.03$) while exhibiting similar treatment related mortality (TRM) rates in both groups [31]. Child et al. came to a very similar finding in the MRC Myeloma VII Trial where myeloma patients (<65 years) either received standard conventional chemotherapy or HD-chemotherapy and ASCT: CR (8% vs. 44%, $p < 0.001$), OS (42.3% vs. 54.1%) and progression-free survival (PFS) rates were significantly in favor of the intensively treated myeloma patients [32].

After publication of these clinical trials, several highly effective anti-myeloma agents have been introduced for treatment of newly diagnosed and relapsed myeloma patients, ushering a new era of anti-myeloma treatment and questioning the

relevance of ASCT in treatment of MM [33]. IMiDs and proteasome inhibitors are the most frequently used agents for induction, consolidation and maintenance therapy ([21, 34–39],) with substantial improvement in CR, OS and PFS rates.

Despite these promising results with the novel anti-myeloma agents, HD-chemotherapy and ASCT is considered gold standard consolidative treatment in transplant-eligible myeloma patients. The novel anti-myeloma agents have not replaced ASCT. In a recent European phase III multicenter, randomized study, Gay et al. analyzed the clinical outcome of myeloma patients undergoing either HDC plus ASCT or conventional chemotherapy plus lenalidomide (for consolidation treatment) followed by maintenance therapy: after a median follow-up of 52 months the study showed that PFS was significantly higher with HD-chemotherapy plus ASCT compared to chemotherapy plus lenalidomide (43.3. vs. 28.6 months, $p < 0.0001$) [40]. Based on current prospective data, the American Society for Blood and Marrow Transplantation (ASBMT) generally recommends an early, upfront ASCT in newly diagnosed MM patients [10].

5.2.3 Single Versus Tandem ASCT

The role of two successive ASCT (tandem) vis-a-vis a single treatment is controversial in patients with multiple myeloma. Attal et al. published the first randomized clinical trial analyzing the efficacy of single versus double ASCT in MM. All together 399 newly diagnosed myeloma patients were enrolled in this study undergoing induction therapy with VAD, single or tandem ASCT, and maintenance therapy with interferon (IFN). The 7-year EFS was 10% in single-transplant group and 20% in tandem-transplant group ($p = 0.03$). The probability of surviving event-free for 7 years after the diagnosis was 10% in the single-transplant group and 20% in the tandem-transplant group ($P = 0.03$). The estimated OS rate after 7 years was 21% in the single-transplant group and 42% in the double-transplant group ($P = 0.01$). Among patients who did not have a VGPR within three months after first transplantation, the probability of surviving 7 years was 11% in the single-transplant and 43% in the transplant-transplant group ($P < 0.001$) [41]. In the Italian Bologna 96 clinical study ($n = 321$), upfront tandem ASCT was superior compared to single ASCT regarding CR or near CR (47% vs. 33%, $p = 0.008$), relapse-free (42 vs. 24 months, $p < 0.001$) and EFS (35 vs. 23 months, $p = 0.001$) in newly diagnosed MM patients [42].

Contradictory results were presented in two meta-analyses analyzing the role of tandem ASCT in MM, revealing that no apparent improvement in EFS or OS was noted by applying tandem ASCT [43–45]. Although not supported and administered by other myeloma treatment centers, we believe that perspective tandem ASCT in the setting of the Total Therapy approach (Fig. 5.2.) is an effective and potent treatment concept leading to cure in a large number of low-risk MM patients [22].

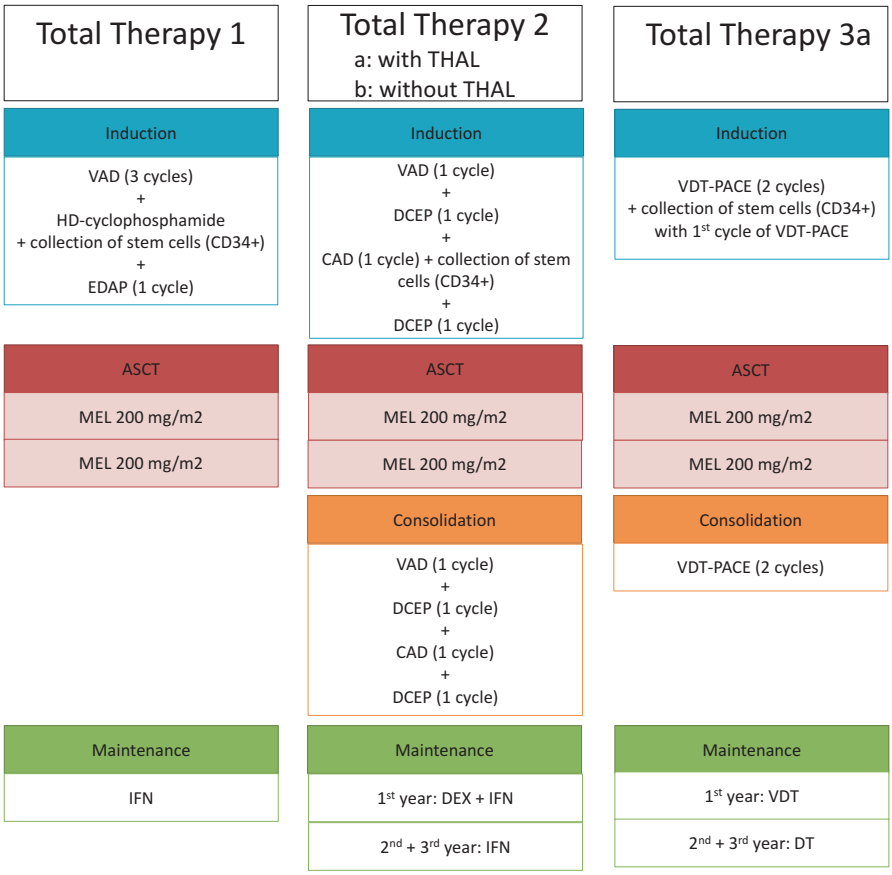

Fig. 5.2. Overview of Total Therapy (TT) trials, TT1, TT2a and b, TT3a. VAD: vincristine, adriamycin, dexamethasone, HD: high-dose, EDAP: etoposide, dexamethasone, adriamycin, cisplatin, MEL: melphalan, IFN: interferon, DCEP: dexamethasone, cyclophosphamide, etoposide, cisplatin, CAD: cyclophosphamide, adriamycin, dexamethasone, DEX: dexamethasone, VDT-PACE: bortezomib, dexamethasone, thalidomide, cisplatin, adriamycin, cyclophosphamide, etoposide, VDT: bortezomib, thalidomide, dexamethasone, DT: dexamethasone, thalidomide

5.2.4 ASCT in Relapsed MM

In relapsed MM, HD-chemotherapy with ASCT is an established treatment regimen that needs to be considered in any physically fit patients. The Scandinavian myeloma research groups have recently published clear evidence underlining the importance of ASCT in the setting of relapsed disease. Patients who relapsed after first HDC and ASCT received either a second ASCT, novel anti-myeloma agents or conventional chemotherapy, respectively: the OS was 4 years in the ASCT-salvage group compared to 3.3 years in group treated with novel drugs ($p < 0.001$) and 2.5 years for those treated receiving conventional chemotherapy ($p < 0.001$) [46]. In the

randomized, open-label, phase III NCRI Myeloma X Relapse trial similar findings were noted, showing that myeloma patients who underwent salvage ASCT had a significant longer median time to progression compared to patients who only received a cyclophosphamide based salvage regimen [47]. Giralt et al. have recently released a consensus expert guideline on behalf of the International Myeloma Working Group (IMWG), and other blood and bone marrow societies (EBMT, ASBMT), indicating that HD-chemotherapy and ASCT should be defined as standard salvage therapy for MM patients who have relapsed after the primary therapy that did not include ASCT. In patients who had ASCT in primary therapy and relapsed after 18 months, HD-chemotherapy and ASCT should be considered as appropriate therapy as well. According to the expert opinion, the conditioning regimen for ASCT in relapsed MM and maintenance therapy after ASCT need to be further investigated in clinical trials, including application of novel agents, such as IMiDs, proteasome inhibitors, and monoclonal antibodies [48].

5.2.5 ASCT in Elderly Patients

For a long period of time it was required that MM patient had to be less than 65 years old in order to apply HD-chemotherapy and ASCT. As MM mainly affects elderly patients, a large number of patients were not considered suitable for HD-chemotherapy and ASCT. However, age above 65 years is no longer an exclusion criterion for application of HD-chemotherapy and ASCT in MM. According to IMWG, elderly MM patients can be divided into frail, intermediate and fit patients, depending on age, comorbidities, cognitive and physical performance status. In conventionally treated elderly MM patients (without ASCT), the 3-year OS was 84% in fit, 76% in intermediate, and 57% in frail subsets [49]. In the last couple of years the utilization of ASCT has markedly increased mainly in the elderly patient population. In a large European study with over 53.000 MM patients who underwent first ASCT between 1991 and 2010 it was clear that application of ASCT has increased in all age groups. However the highest increase was noted in patients above the age of 65: from 1991–1995 only 3% received ASCT while 15 years later, from 2006–2010, 18.8% underwent ASCT. The median 2- and 5-year post-ASCT survival has considerably increased in elderly patients (≥70 years) to 80.2% and 49.7%, respectively [50]. The continued usage of ASCT in elderly patient is being challenged with the arrival of novel agents that provide excellent clinical outcome with adequate tolerability. In their randomized study, Facon et al. demonstrated that melphalan plus prednisone and thalidomide (MPT) was associated with significantly better OS than HDC with melphalan (100 mg/m^2) in elderly patients, 51.6 vs. 38.3 months respectively ($p = 0.027$) [51]. HD-chemotherapy with melphalan 200 mg/m^2 followed by ASCT is associated with high mortality rate in elderly patients [52]. It has been shown that with 30% dose reduction of the conditioning regimen, HD-chemotherapy and ASCT can be safely performed. HD-melphalan with only 140 mg/m^2 based ASCT is less toxic and appears to be equally effective as melphalan 200 mg/m^2 in elderly patients [52]. Badros et al. demonstrated that with dose reduction of the conditioning regimen even tandem ASCT is possible in newly diagnosed and pretreated, elderly myeloma patients [52].

5.3 Mobilization and Collection of Peripheral Blood Stem Cells

It is general consensus that circulating peripheral CD34+ blood stem cells PBSC are collected and used for ASCT in patients with MM. According to the IMWG the mobilization of peripheral stem cells in myeloma patients should be performed in an early stage of the disease, preferably within the first four cycles of induction therapy with novel anti-myeloma agents [43, 44]. The importance of collection of appropriate amount of hematopoietic stem cells to perform a second ASCT during the early course of the disease cannot be understated [48]. In general, increasing age, more than 12 months of anti-myeloma treatment prior to stem cell collection, less than 200 × 10(9)/l platelets prior to mobilization, and mobilization with growth factors only were correlated with negative CD34+ yield [53].

G-CSF alone or cyclophosphamide (C) plus granulocyte-colony stimulating factor (G-CSF) are the most widely used stem cell mobilization regimen for patients with MM [54, 55]. In a randomized phase II trial 34 patients were treated with C + G-SCF (cyclophosphamide 2 g/m^2) (arm A) and 35 with G-CSF alone (arm B). In arm A 94% reached the goal of stem cell collection of at least 3 × 10(6)/kg, in arm B it was only 77% ($p = 0.084$). The median number of apheresis needed was significantly lower in arm A than in arm B (1 vs. 2, $p = 0.035$), and two patients required plerixafor in arm A and five in arm B [54]. In the Total Therapy trials it is shown that DT-PACE (dexamethasone, thalidomide, cisplatin, adriamycin, cyclophosphamide and etoposide), VDT-PACE (plus bortezomib) and M-VDT-PACE (plus melphalan) are considered excellent mobilizing regimen for collection of CD34+ cells [53, 56, 57].

In 2008 the U.S. food and drug administration (FDA) approved the application of plerixafor in combination with G-CSF for mobilization of hematopoietic stem cells to the peripheral blood for collection and subsequent autologous transplantation in MM. Plerixafor antagonizes the binding of the chemokine stromal cell-derived factor-1 (SDF-1) to its related receptor CXCR4 which leads to a rapid and reversible mobilization and release of hematopoietic stem cells into the peripheral blood [58]. Afifi et al. have demonstrated that the addition of plerixafor to C + G-CSF was associated with higher success of SC collection, less toxicities and less financial burden compared to C + G-CSF alone, mainly due to lesser rate of hospitalization, decreased rate of salvage mobilization, and decreased G-CSF usage [54].

5.4 Total Therapy in MM

The Total Therapy (TT) concept was introduced at our institution by Bart Barlogie 30 years ago to overcome refractoriness and relapsing course of MM. The main goal was to give all available anti-myeloma agents upfront in order to achieve synergistic impact and address clonal heterogeneity and drug-resistance in myeloma cells in order to prevent later relapse. The backbone of each TT trial is the application of two successive ASCTs in the framework of induction, consolidation and

maintenance therapy (Fig. 5.2.). In the first TT1 trial started in 1990 only conventional chemotherapy was used, in TT2(a) consolidation therapy (after tandem ASCT) and in TT2(b) trial thalidomide, and later on in TT3(a) bortezomib was introduced (Fig. 5.2.). It was shown long time before novel agents were used that long-term CR and cure is an achievable goal in myeloma patients [59]. After 14 years of follow-up in the TT1 trial where 231 patients were enrolled, 23 patients remained alive without any progression with a 14-year plateau in OS [11, 12]. The incorporation of consolidation therapy in the post-transplant setting in TT2(a), thalidomide in TT2(b), bortezomib in TT3(a) further improved the complete response duration, EFS and OS rate, respectively (Fig. 5.2.) [60]. The TT clinical trials clearly demonstrate that a certain portion of MM patients are curable. The 10-year PFS and CR increased from 8.8% and 17.9% in TT1 to 15.5% and 28.2% in TT2(a) to 25.1 and 35.6% in TT2(b) and to 32.9% and 48.8% in TT3(a), respectively [22]. TT4, TT5 and TT6 are ongoing clinical trials analyzing clinical outcome in low-risk, high-risk and pretreated MM patients, respectively.

5.5 Allogeneic Stem Cell Transplantation

Allogeneic stem cell transplantation (Allo-SCT) is not a standard treatment option for patients with MM because of its high treatment-related mortality rate [61]. However, because of the curative graft-versus-myeloma effects, it should be considered in selected patients with early relapse (<24 months) after primary therapy that included ASCT, patients with high risk characteristics defined by cytogenetic/FISH, presence of extramedullary disease and/or plasma cell leukemia, respectively [48, 62]. The Dutch myeloma research group has recently shown that Allo-SCT can produce long-term favorable clinical outcome in HRMM patients with CR rate of 48.3%, PFS of 30.2 months and 10-year OS of 51%, respectively [63]. However, in the relapsed/refractory setting the clinical outcome was poor, in particular in patients who relapsed within 18 months after ASCT.

In recent years Allo-SCT with reduced-intensity conditioning (RIC) has been increasingly used to treat patients with myeloma. This treatment option is associated with lower toxicity and substantial decrease in the incidence of transplant-related mortality rates. However, in a recently published study by the European Group for Blood and Marrow Transplantation (EBMT) in more than 400 relapsed and progressive MM patients after prior ASCT (>2 ASCT: 44.6%) who underwent related or unrelated RIC-Allo-SCT it was shown that median OS was 27.7% with a PFS of 9.6% and a non-relapse mortality (NRM) rate of 21.5%. In a multivariate analysis it was demonstrated that CMV seronegativity of both donor and patient was associated with significantly better PFS, OS and NRM. Moreover, OS was better in patients who had less than two prior ASCT, and NRM rate was lower in patients who underwent RIC-Allo-SCT in shorter time from the first ASCT [64].

An evolving concept is the performance of an ASCT/Allo-SCT successively in a tandem approach. In a recently performed large Japanese matched-pair analysis no

significant differences in OS were noted between patients who underwent tandem ASCT and ASCT/Allo-SCT [65]. In contrary, long term results generated by the EBMT non-myeloablative allogeneic stem cell transplantation in MM (NMAM) showed that PFS and OS were significantly favorable for patients who received ASCT/RIC-Allo-SCT compared to those who underwent tandem ASCT, with OS and PFS rates at 96 months of 49% versus 36% (p = 0.03) and 22% versus 12% (p = 0.027), respectively [66]. The corresponding progression and/or relapse rate was significantly lower in RIC-Allo-SCT (60%) compared to the tandem (82%) group (p = 0.002), but with higher rate of 36-month non-relapse mortality rate of 13% and 3% (p = 0.0004%), respectively.

Despite promising data, it is general practice that Allo-SCT in MM patients should be performed in clinical trials to better define the role of salvage allogeneic SCT after primary therapy. Furthermore the post-allogeneic SCT maintenance therapy needs to be explored in prospective trials, and the role of salvage allogeneic HCT in patients with MM relapsing after primary therapy needs to be defined [48].

5.6 Conclusion

The application of HD-chemotherapy and ASCT is considered standard consolidation treatment in patients with MM. Although several novel anti-myeloma agents have been introduced in the last couple of years, HD-chemotherapy and ASCT remains an integral part and solid backbone in treatment of MM. Cure in MM can be achieved in a large number of low-risk patients by applying HD-chemotherapy and tandem ASCT in the setting of the Total Therapy approach. HD-chemotherapy and ASCT can be effectively used in refractory and relapsing myeloma patients, as well as in selected elderly myeloma patients.

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Chapter 6
Simultaneous MRI and 18F-FDG-PET Imaging in Multiple Myeloma: A Model for Evaluation of the Disease and Therapeutic Changes in SCID-hu Mice

Tarun K. Garg and Tarun Pandey

Abbreviations

FDG-PET	^{18}F–Fluorodeoxyglucose-positron emission tomography
ASCT	Autologous stem cell transplantation
BLI	Bioluminescence imaging
CAR-T	Chimeric antigen receptor-T
CT	Computed tomography
DCE-MRI	Dynamic contrast-enhanced MRI
ELISA	Enzyme-linked immunosorbent assay
Exp-NK	Expanded natural killer
hIg	Human immunoglobulins
iNKT	Invariant natural killer T cells
MRI	Magnetic resonance imaging
MGUS	Monoclonal gammopathy of undetermined significance
MM	Multiple myeloma
NOD/SCID/IL2Rγ^{null}-Hu	Non-obese diabetic-SCID-IL2 receptor gamma null-human
PET	Positron emission tomography
SCID	Severe-combined immunodeficiency

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6.1 Introduction

MM is a malignancy of terminally differentiated B cells characterized by a clonal proliferation of plasma cells. The plasma cells accumulate in the bone marrow; produce lytic bone lesions and excessive amounts of monoclonal proteins (usually IgG or IgA type or free light chain) [1]. According to The American Cancer Society, currently there are estimated 88,490 people living with myeloma in the United States. Approximately 30,330 new cases will be diagnosed and about 12,650 deaths are expected to occur in the year 2016 with this disease in US alone. MM is preceded by a premalignant stage called monoclonal gammopathy of undetermined significance (MGUS) and asymptomatic smoldering MM. Rajkumar et al. have elegantly summarized the diagnostic criteria of the International Myeloma Working Group for premalignant and malignant MM [2]. The diagnosis is based on laboratory parameters in combination with bone marrow biopsy or bone marrow aspiration which provide information about paraproteinaemia, plasma cell infiltration and osteolytic bone destruction [3]. Approximately 80% patients present lytic bone lesions with a high risk of pathological fractures, hypercalcaemia and bone pain.

Despite the major advancements in progression-free and overall survival with the introduction of novel agents such as bortezomib and lenalidomide, the majority of MM patients relapse, likely due to the outgrowth of refractory myeloma cells and the disease remains essentially incurable [4]. Autologous stem cell transplantation (ASCT) has been considered frontline therapy in newly diagnosed myeloma patients; however, a range of combinations of novel drugs with ASCT, in a sequential treatment approach have recently become available, creating new opportunities for clinical investigations [5]. The role of immunotherapy is increasingly recognized in myeloma. A phenomenal number of immunostimulatory compounds, antibodies, vaccines, for myeloma are in different stages of the development in either animal model or clinical trials in conjunction with different agents. More recently, two new monoclonal antibodies, daratumumab and elotuzumab, were approved by the U.S. Food and Drug Administration (FDA) for myeloma. Furthermore, cellular therapies such as dendritic cells, invariant natural killer T (iNKT) cells, natural killer (NK) cells, chimeric antigen receptor-T (CAR-T) cells are also being evaluated in different malignancies including myeloma. These therapies seem to have great potential for long-term disease control and may be translated into the personalized cellular therapy [6, 7].

One of the important challenges while dealing with tumors like multiple myeloma is reliable real time assessment of tumor burden in response to different treatments. Conventional radiography is a common technique that has been used in lab and clinical setting for over 40 years for the real time assessment of tumor burden; however, it has several limitations [8, 9]. Another commonly used approach to overcome this challenge is luciferase transfection. This technique is used on the established myeloma cell lines that are easy to transfect with luciferase and the tumor load is assessed by live bio-imaging. However, primary myeloma cells, which are generally available in limited numbers, survive poorly in vitro, *and are* extremely difficult

and labor intensive to attempt luciferase transfection. Although enzyme-linked immunosorbent assay (ELISA) for human immunoglobulins (hIg) in murine sera can be used for measuring myeloma burden (provided non-secretory myeloma cases are not utilized), a non-invasive and real time method of assessing tumor involvement would certainly be of advantage. In this respect MRI combined with FDG-PET imaging can be of immense potential. While MRI can help determine the anatomical locations; extent of tumor involvement and tumor burden FDG-PET scans are sensitive for early tumor detection, evaluation of recurrence and provide biochemical information concerning tumor metabolism, replication rate, ischemia levels, and other physiological information.

In the following sections we will discuss role of these techniques in evaluation of myeloma tumor burden and shall focus on our experience about live imaging using MRI/PET for the assessment of tumor burden after ENK-cell therapy in a mouse model for myeloma and how this technique can be successfully used for drug-related and/or cytotherapy-associated evaluation in small animals.

6.2 Current Methods for Assessment of Tumor Burden in Murine Model of hu-myeloma

Animal models have played a major role in understanding many physiological, biochemical processes and different pathways involved in the development of cancer [10]. These in vivo models mimic the human disease closely, and allow testing new therapies and target orientated drug screening [11]. In spite of these animal models providing significant information regarding drug efficacy, several factors should be considered while inferring mouse data to the clinical testing [10]. Monitoring lifespan of animals and caliper-based measurements are commonly used for the response of therapeutic efficacy and target-drug interactions in subcutaneous tumor xenograft models [12, 13]. However, measuring tumor size with calipers has several limitations, such as, not providing the internal structure and the cellular heterogeneity of the tumor. Furthermore, therapeutic agents having cytostatic effect, but not cytotoxic, may not result in decrease in tumor size, thus leading to miscalculation of the effect of therapeutic agents [14]. Regardless, caliper measurements may not be a good option for mouse-human myeloma models since myeloma grows in the protective bone microenvironment.

6.2.1 Murine-Human Model for Myeloma

The xenograft models of MM, in which human MM cells grow in immunocompromised mice, have been beneficial in optimizing drug schedules and doses and have undoubtedly benefited the MM patients [15]. Myeloma cell lines or primary myeloma cells are grown within human fetal bone, implanted subcutaneously in

severe-combined immunodeficient (SCID) or non-obese diabetic-SCID mice with a defect in the IL2 receptor gamma chain (NOD/SCID/IL2Rγ^{null}-Hu). Yaccoby et al. (1998) has described SCID-hu model in detail [1]. Since SCID mice they are devoid of inherent immune cells, these allow the engraftment of human fetal bone and human myeloma. Primary myeloma cells do not survive once removed from the patient but grow in the human bone fragment in SCID-hu, remain restricted to the human bone microenvironment and manifest typical myeloma disease symptoms. Newly formed blood vessels at the myeloma tumor site also originate from human cells and create human bone microenvironment.

NOD/SCID/IL2Rγ^{null} mice have distinct advantage for the study of cellular and immunotherapy and ensure that adoptively transferred cells are not rejected [16]. After the inoculation of myeloma cells, tumor growth can be monitored by a rise in the host's serum level of human immunoglobulins (hIg) of the M protein isotype by ELISA, changes in bone calcification by conventional radiography, and by bioluminescence imaging if luciferase transfected myeloma cells have been used.

6.2.2 Radiography and ELISA

Conventional radiography is a low-cost and fast imaging option but has several limitations such as low sensitivity to early osteolytic lesions, as lytic lesions are apparent only after 30–50% of bone mineral density is lost (Fig. 6.1). In addition, diffuse bone marrow involvement that may not be associated with significant decrease in density or bone destruction, is not readily detected.

Durie and salmon in 1975 introduced a staging system in myeloma patients, based upon M-protein level in serum and urine, which correlates well with myeloma cell mass. This staging system serves as a gold standard in clinical practice [17]. Studies have shown that circulating hIg levels represent the tumor burden in myelomatous mice (Fig. 6.2) and are routinely performed for the evaluation of growth in tumor burden, pre and post-therapy [1, 18]. The levels of human IgG, IgA, $\varkappa$, and λ light chains are determined by ELISA. However, there are certain disadvantages in this approach as the kinetics of the increased hIg levels varies among patients'-derived myeloma cells and there is a lack in correlation between the time of detection of hIg and the number of myeloma cells inoculated, marrow plasmacytosis or other patient characteristic [1]. This implies that any real time monitoring of myeloma based on M-protein levels is only good for a particular patient or class of myeloma cells and cannot be generalized across the board. It also implies that if the clonal population is altered during the course of the disease then the results may not be valid. Further, M-proteins alone cannot predict survival or treatment response accurately, since large quantities of M-protein can be produced by small number of myeloma cells and vice versa. In addition, this approach may not be useful if the cells used for inoculation are from non-secretory myeloma patient that constitute approximately 3% of all myeloma patients.

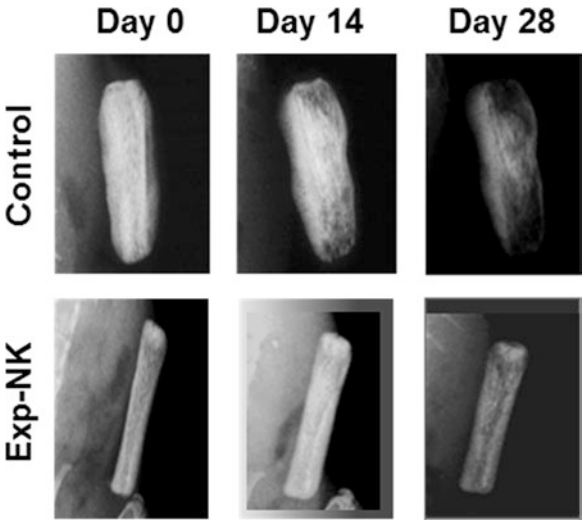

Fig. 6.1 X-radiographs showing loss in the density of human fetal bone in myeloma-bearing mouse. NOD/SCID/IL2Rγ^{null} mice after implantation of human fetal bones were engrafted with OPM2 myeloma cells. Bone resorption can be seen in the untreated control mouse (*upper panel*) as the time progressed, compare to the Exp-NK treated mouse (*bottom panel*)

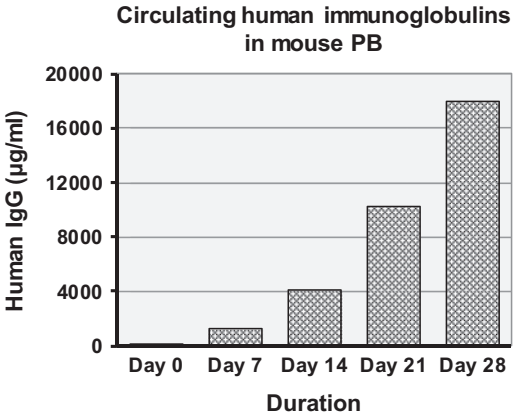

Fig. 6.2 Human-Ig levels in NOD/SCID/IL2Rγ^{null}-Hu model. Levels of circulating hIg determined by ELISA from the mouse blood after inoculation of OPM2 myeloma cells showed an increase in tumor burden with the progression of time

6.2.3 Bioluminescence Imaging

In recent years, bioluminescence imaging (BLI) has drawn much attention and is commonly used in the preclinical drug development. It allows gaining insight in the engraftment pattern, growth dynamics and therapy-related changes in the tumor volume following treatment of xenograft tumors in mice (Fig. 6.3). In BLI genetically modified cells are transfected to express luciferase, which converts luciferin to oxyluciferin in the presence of oxygen and emits photons [19]. Luciferase-derived photon emission can be detected externally from the cells located several millimeters below the skin [20]. Further, luciferase and BLI do not affect the tumor growth in vitro or in vivo and is efficiently used in cancer imaging [21]. In addition, BLI can detect as few as 500 cells at specific anatomical sites in vivo and allows

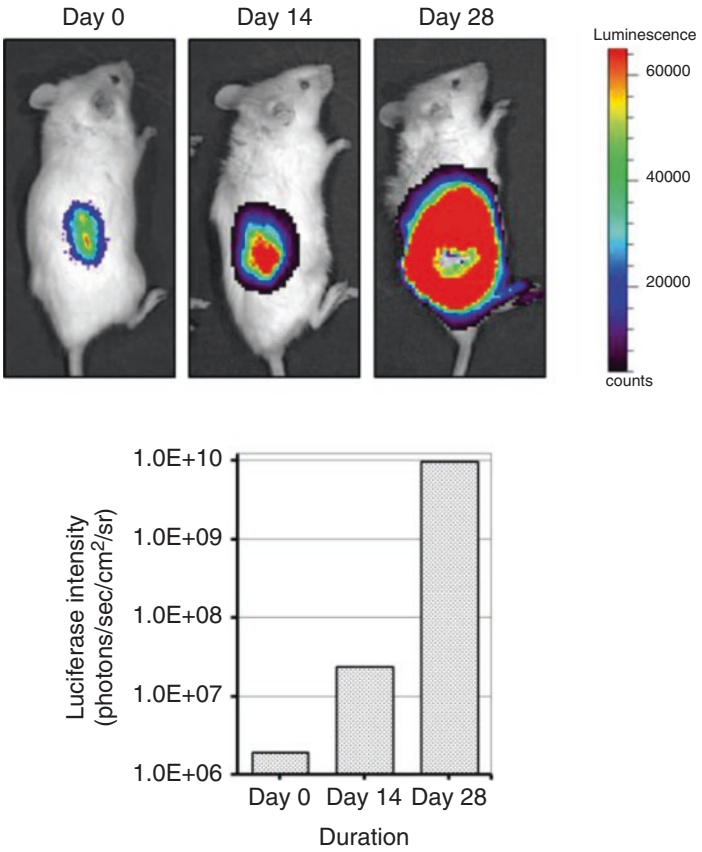

Fig. 6.3 Bioluminescent imaging of myeloma growth in vivo. Luciferase-transfected OPM2 myeloma cells were engrafted in the NOD/SCID/IL2Rγ^{null}-hu mice. Weekly assessment by live animal imaging shows a rapid growth of myeloma tumor (*upper panel*). Bioluminescence intensity was analyzed immediately and depicted in photons/second/cm^2/steradian (*lower panel*). Bioluminescence intensity was increased with time and was highly correlated with increased circulating hIg (Fig. 6.2)

quantification of the tumor burden in humanized models (Fig. 6.3) [18, 22]. A decrease in emission of photons is attributed to the cytotoxic effects of the drug/therapy as a result of either induction of cell death or a reduction in cell metabolic ability. BLI has been used in a large number of preclinical, cancer pharmacology and immunotherapeutic studies such as brain, breast, and lung carcinoma, sarcomas including multiple myeloma [18, 23]. Recently, we used BLI and showed significant inhibition in myeloma tumor growth in NOD/SCID/IL2Rγ^{null}-Hu mouse model after ENK cell therapy, which successfully lead the way for clinical studies in

high-risk myeloma at our center [18]. Unfortunately, this technique also has some limitations. The intensity of photon emission signal can drop or plateau in advanced tumors [24, 25]. This could be due to accumulation of biochemically inactive necrotic tissue in large tumors that contributes to the tumor mass but is unable to metabolize luciferin, causing discrepancy between the tumor size and bioluminescence output [25]. Further, dominant signals produced at one location/organ can mask a weaker signal produced by another metabolically active region [26]. Another drawback in this approach is that primary myeloma cells do not grow in vitro, are difficult to transfect with luciferase, henceforth cannot be used for BLI.

6.2.4 Magnetic Resonance Imaging

Magnetic resonance imaging (MRI), also known as nuclear magnetic resonance imaging, is based on the absorption and emission of energy in the radio frequency range of the electromagnetic spectrum [23]. Water molecules and fat in the body contain hydrogen atoms. The nuclei (protons) of these atoms become aligned under a very strong magnetic field (about 0.2 to 7 Tesla) and behave like tiny magnets or dipoles processing along the axis of the main magnetic field (spins). The precession frequency depends on the magnetic field strength. An external radiofrequency (RF) pulse matching the precession frequency of the spins is used to impart energy to these spins (resonance), following which the spins flip the direction of precession and become in sync with one another. This creates a rotating magnetic vector that emits energy and is capable of producing a radio signal, which is measured by receivers in the scanner to create an image. Ultimately the protons gradually return to their normal alignment once the RF pulse is turned off.

MRI is a versatile technique for the quantification of tumor volume and to address tumor physiology in small animal studies [27]. It provides high spatial resolution [28] and good soft tissue contrast. Its ability to integrate anatomical and functional information provides great insights into the disease processes, including cancer [29]. Further, MRI allows for repeat imaging and follow-up without any exposure to radiation. Several researchers have demonstrated the utility of MRI in experiments involving small animals. MRI has been successfully used to demonstrate the infiltration of intraprostatic gene therapies in a mouse model of prostate cancer [30] as well as delayed tumor growth in a mouse model of orthotopic glioma after suicide gene therapy [31]. With MRI, we observed early changes in intracortical and trabecular regions of the bone, studied the intramedullary and extramedullary tumor growth patterns that matched with radiographic lytic lesions in human fetal bone component in NOD-SCID/IL2Ry^{null}-hu mice after inoculation of myeloma cells (Figs. 6.4 and 6.5). We also demonstrated regression of tumor and return of normal

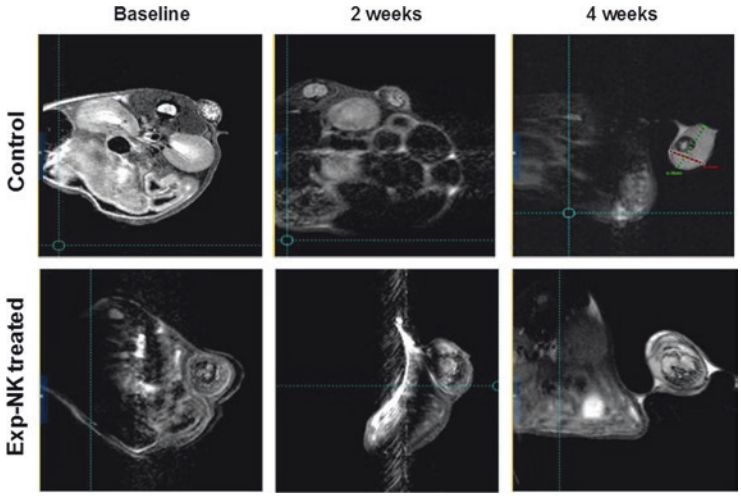

Fig. 6.4 MRI visualizing tumor growth and effect of Exp-NK cells in myelomatous mouse. NOD/SCID/IL2Rγ^{null}-hu mice were engrafted with OPM2 myeloma cells and treated with 160 M Exp-NK cells. The mice were imaged to determine the baseline, after 2–4 weeks for myeloma growth (Control, *upper panel*) and evaluated for Exp-NK cell therapy-mediated changes (*bottom panel*). In control, an enlarged extramedullary tumor is observed with good soft tissue contrast compare to Exp-NK treated group where the bone integrity is well protected with a significantly reduced tumor

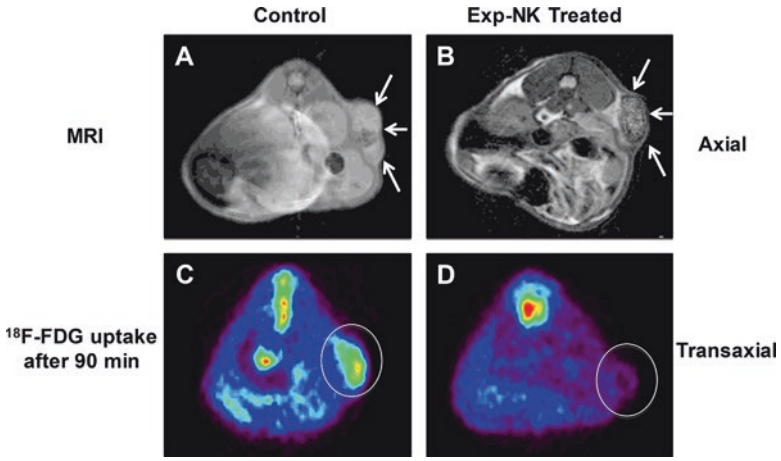

Fig. 6.5 Inhibition of primary myeloma growth visualized by MRI and FDG-PET scan**.** NOD/SCID/IL2Rγ^{null}-hu mice were engrafted with primary myeloma cells. The mice were scanned for the tumor load by MRI and metabolic activity by FDG-PET imaging. (**a**) MRI revealed macroscopic morphology, gross loss of bone and enlarged tumor mass in the control mouse. (**b**) Mouse received 160 M expanded NK cells maintained the bone structure with no visible tumor mass compare to the control mouse. (**c**) High ^{18}F–FDG uptake in control mouse confirmed higher tumor load by FDG-PET imaging. (**d**) No active tumor was detected in Exp-NK cell treated mouse

bone integrity after successful ENK cell therapy in myelomatous mice (Fig. 6.4). Although MRI is a useful imaging technique, but this technology has some disadvantages such as low sensitivity for detection of very early disease activity compared to functional imaging modalities, longer acquisition time and high costs. Also, presence of indwelling metal devices such as identification microchips in the body limits its utility for preclinical studies [32–34]. In addition, the detection of lesions in the bone implants can be challenging because of the respiratory movements in small animals.

Use of MRI contrast agents and higher magnetic fields has improved the sensitivity of MRI [29]. Recently, MRI has been used with drug-containing liposomes, either labelled with gadolinium or other paramagnetic substances, to facilitate image-supervised therapeutic delivery and subsequent monitoring of efficacy [35, 36]. Advancements in technology have now led to more sensitive quantitative MRI techniques, such as high-field MRI and dynamic contrast-enhanced MRI (DCE-MRI), which are even more valuable for research into tumor vasculature and the effects of drugs [23].

6.2.5 ^{18}F–FDG-PET Imaging

^{18}F–Fluorodeoxyglucose (FDG), a radiolabeled analogue of glucose, is introduced into the animals and its uptake and metabolism is monitored. The cells with high metabolic rate take up F^{18}-FDG in proportion to their metabolic activity. The ^{18}F-FDG gets trapped within the cell after phosphorylation. The ^{18}F decays via emission of positrons. Immediately after its decay annihilation reaction occurs and the positron combines with an electron to generate two 511 KeV photons. These are detected using the coincidence detection circuitry within a PET scanner. A three-dimensional image is created of the functional processes corresponding to the biological activity and uptake of the radiotracer. It presents crucial insight into the biology of the system [37]. High uptake indicates high glucose metabolism and intensified glycolysis that is associated with malignancy which helps in differentiation between malignant and non-malignant tissue [38, 39].

One advantage of FDG-PET is the whole body can be imaged in a single procedure, in approximately ~45 min [32, 40]. FDG-PET has been sensitive in detecting the areas which could not be imaged by MRI. Further, PET can detect bone marrow involvement with high sensitivity and specificity. In addition, it can differentiate between extramedullary and intramedullary lesions [40]. A major advantage of FDG-PET is probably its ability to detect disease function, which can discriminate between inactive and active state of the disease [41].

FDG-PET has strong potential in translational research for therapy-related assessment in the tumor volume, proliferation and metabolism and drug biodistribution in laboratory animals [42, 43], all of which play an important role in establishing drug efficacy in various malignancies [44–48]. During preclinical stage of ENK cell therapy program against myeloma, we observed considerably high uptake in myeloma both within and outside of the human fetal bone implanted in NOD-SCID/IL2Ry^{null}-hu mice compared to the mice received ENK cells (Fig. 6.5). However, an obstacle associated with the heterogeneity of glucose uptake in various areas of a tumor was reported for PET in preclinical studies which could not be correlated with standard caliper assessments to assess the antitumor activity of enzastaurin, a novel protein kinase C-beta II inhibitor in mouse xenografts [49]. Unfortunately, due to the spatial resolution limits of the PET scanner, in FDG-PET imaging, small lesions (<5 mm) may go unnoticed and may provide false-negative results. [17]. Likewise, metabolically active areas after inflammation or infection or from brown fat may show increased activity leading to false-positive results [50]. In addition, the FDG-PET imaging system for small animals is expensive, and is mainly restricted to bigger research centers [23].

Despite these limitations, FDG-PET imaging in small animals is a viable option which allows therapeutic interventions and optimization of treatment, one of the main goals of preclinical studies.

6.3 Conclusion

Circulating hIg and x-radiography has been conventionally used for the assessment of tumor burden in mice for preclinical studies in myeloma research. However, there are drawbacks associated with these approaches. Though, BLI provides the engraftment pattern, growth dynamics and therapy-related changes in tumor volume following treatment in xenograft models but it needs genetic modification of the cells, which is a challenge, for primary myeloma cells. Many of these limitations could be addressed by adoption of novel, non-invasive molecular imaging techniques such as MRI and FDG-PET. Although, MRI and FDG-PET involves high cost, availability and requirement of a radio-nucleotide facility, these are useful adjunctive imaging methods, established to evaluate therapeutic response in preclinical studies. Major advantage and disadvantages for these techniques have been summarized in Table 6.1.

Table 6.1 Advantages and disadvantages of different techniques commonly used for the evaluation of myeloma tumor burden in laboratory mice

Modality/technique	Advantages	Disadvantages
ELISA (hIg)	• Cost effective • Quantitative • Prognostic marker for the disease • Historical use/validated • Sophisticated equipment not required • Pre- and post-treatment assessment	• Labor intensive • Involve multiple bleeding of mice • Usually done at the end of the experiment after establishing baseline before starting therapy
Radiography	• Easy accessibility • Cost effective • Historical use/validated	• Poor sensitivity • Limited to bony defects • Early events are not evident • Captures only advanced bone damage –Possible need for repeat images
Bioluminescence imaging (BLI)	• Wide applicability • Simultaneously monitor several molecular events • Relatively inexpensive • Amenable to smaller research laboratories	• Requires genetic manipulation of investigated cells • Challenging to transfect primary tumor cells • Provides limited anatomical information • Reduced sensitivity with increased imaging depth
MRI	• No radiation exposure • Lesion number has prognostic significance • Images extra medulary disease (EMD) • High spatial resolution • Good soft tissue contrast • Provides both anatomical and functional information	• Long acquisition time • High cost • Requires expensive equipment • Low sensitivity • Interference with metal objects/ identification chips (generally used in small animals) • Over-representation of osteolytic lesions – Bone infiltration may be misinterpreted as osteolytic lesion • Limited imaging field, subject to motion artifact
FDG-PET	• High sensitivity • Provides biochemical information • Three-dimensional imaging • Monitor changes in tumor metabolism and drug biodistribution • Prognostic significance pre- and post-treatment • Novel radioisotopes may offer additional disease-relevant information	• High cost • Limited availability, requires specialized equipment • Limited anatomical information • Relatively long acquisition time • Requires radio-nucleotide facilities

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Chapter 7
The Emerging Role of Cardiac Stem Cells in Cardiac Regeneration

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7.1 Introduction

Heart diseases are one of the leading causes of death and disability worldwide. These diseases lead to loss of cardiac tissue through death of myocytes by apoptosis and necrosis. The successive progression of heart disease involves loss of the myocardium, scar formation and remodeling of the remaining cardiac tissue. The average left ventricle contains approximately four billion cardiomyocytes and the myocyte loss in infarction-induced heart failure is about one billion. After infarction, the remaining myocytes are unable to restore the host tissue, and the injured heart worsens functionally with time. Current therapeutic approaches available including medical therapy, mechanical left ventricular assist devices, and cardiac transplantation are primarily focused at limiting disease progression rather than repair and restoration of healthy tissue and function. The limited efficacy and comorbidity of these current treatments have thus stimulated the interest to investigate other alternative and additional long-term curative measures. In this context, cardiac stem cell-based therapies have gained significant impetus and seem to hold a great promise for eliminating the underlying cause of the disease by reconstituting lost myocardium with a new network of functional cardiomyocytes.

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7.2 Cardiac Stem Cells (CSCs)

Beltrami et al gave the first report of these endogenous regenerating myocardial stem cells in rats in 2003 [1]. This group showed the existence of Lin^{-} c-kit^{+} (Lin^{-}: negative for various cell specific lineage markers and $ckit^{+}$: positive for ckit, a transmembrane receptor for stem cell factor) cells located in the adult rat myocardium that were positive for Ki67, a mitotic marker and GATA4 and Nkx2.5, transcription factors associated with early cardiac development. In vitro, these cells demonstrated self-renewal capability and also the capacity to differentiate into myocardial, endothelial, and smooth muscle cell lineages. Most strikingly, transplantation of these cells into the hearts of syngeneic rats post myocardial infarction (MI) reduced the extent of myocardial damage compared with controls, and the transplanted cells were able to give rise to myocyte, endothelial, and smooth muscle cell lineages in vivo. Thus, these cells were shown to have all the characteristic features of putative cardiac stem cells. After initial report of rat CSCs, c-kit-positive CSCs have been identified in mice [2], dogs [3] and now also in human beings [4]. All these reports have unequivocally documented that c-kit^{+} CSCs are self-renewing, clonogenic and multipotent both in vitro and *in vivo*. The c-kit^{+} CSCs are distributed throughout the left and right ventricle but tend to concentrate in the atria and apex, since these anatomical areas are exposed to low levels of hemodynamic stress. For details about the niche of CSCs, the readers may refer to Leri et al. [5]. Regarding the origin of these cells, some studies have reported that c-kit+ CSCs originate from the developing heart [6]. On the other hand, contrastingly, several studies have suggested that c-kit+ cardiac cells are mobilized from the bone marrow (BM) and recruited to the heart following injury, and hence may have an extra-cardiac origin [7].

In addition to c-kit, other specific phenotypic markers define other "types" of CSCs in the myocardium, although some of these markers may be co-expressed by some other cells. Various reported populations of CSCs include (1) side population cells that are known to express abcg2, an ABC transporter that effluxes dyes, similar to mdr1; (2) CSCs expressing stem cell associated-marker stem cell antigen-1 or Sca-1+; CSCs expressing (3) CSCs expressing transcription factor Islet1 or Isl1+; CSCs expressing and (4) CSCs derived from cardiospheres or CDCs migrate out of cardiac explants and grow as 3D multicellular clusters [8] (Table 7.1). On the basis of their differentiation property, immature cardiac cells have been classified into four classes: CSCs, progenitors, precursors, and amplifying cells. The first three cell types express c-kit, mdr1/abcg2, and Sca-1, whereas the last type no longer expresses these antigens [19]. It has been reported that Notch1 regulates the transition of CSCs from the primitive immature phenotype to that of amplifying myocytes. Activation of the Notch1 pathway up regulates the expression of Nkx2.5 that drives the differentiation of CSCs to the myocyte lineage. Activation of Notch 1 is also known to enhance the proliferative phase of cardiomyocytes, favor their survival and extend their lifespan both in vitro and in vivo [5]. An alternative source of CSCs has been identified in the epicardium. Epicardium-derived progenitor cells (EPDCs) express Wt1, an embryonic epicardiac gene and exhibit characteristics of CSCs

Table 7.1 Phenotype of different reported populations of cardiac stem cells

S. no.	CSC population	Source	Phenotype	References
1.	c-kitpos CSCs	Myocardium	CD34neg, CD45neg, Sca-1pos, Abcg2pos, CD105pos	[1, 9]
2.	Sca1pos CSCs	Myocardium	CD34neg, CD45neg, FLK1neg, c-kitlow, GATA4pos, NKX2-5low, MEF2Cpos	[10, 11]
3.	Side population cells	Myocardium	CD34pos, CD45pos, Abcg2pos, Sca1pos, c-kitpos, NKX2-5neg, GATA4neg	[12]
4.	Isl-1pos cardiac progenitor cells	Myocardium	CD31neg, Sca1neg, ckitneg, GATA4pos, NKX2.5pos	[13, 14]
5.	Cardiosphere-derived cells	Myocardium	CD105pos, CD34pos, CD45pos, Abcg2pos, Sca1pos, c-kitlow	[15, 16]
6.	Epicardium-derived progenitor cells	Epicardium	Wt1pos, NKX2.5pos, Isl1pos, ckitneg, Sca1pos	[17]
7.	Colony-forming units - fibroblasts	Epicardium	Sca-1pos, PDGFRαpos, CD31neg, c-kitlow, CD45neg, FLK1neg, CD44pos, CD90pos, CD29pos, and CD105pos	[18]

[17]. EPDCs have been shown to contribute to coronary vascular smooth muscle cells and fibroblasts but whether they also differentiate to cardiomyocytes remains controversial. Recently, a population of EPDCs with mesenchymal-like properties, termed as colony-forming units—fibroblasts has been characterized in the developing and adult mouse heart [18]. Their role in cardiac repair and homeostasis, however, still needs to be investigated (Table 7.1).

Given the multiple cell surface markers that have been associated with CSCs, it is discernible that CSCs is a heterogenous group of cells that includes early uncommitted cells and lineage-committed cells, quiescent and activated cells, cycling and non-cycling cells.

7.3 CSCs and In Vivo Fate-Tracing Studies

The identification of multiple putative CSC populations raises the question of whether any of them also participates in cellular homeostasis of the myocardium throughout life and its repair in response to damage in vivo. Many studies have investigated the contribution of endogenous CSCs towards cardiomyocyte renewal in vivo through genetic fate-mapping studies, albeit with varying results. In these studies, the expression of a fluorescent reporter gene is placed under the control of promoters coding for myocyte and vascular proteins to track the origin and fate of cells in experimental animals.

According to one set of these studies, new cardiomyocytes are generated by endogenous CSCs only during myocardial injury and not during normal physiological wear and tear of heart cells. In this regard, Hsieh et al. [20] used an elegant inducible cardiomyocyte-specific transgenic mouse fate-mapping approach to determine the frequency with which cardiomyocytes are refreshed from stem or precursor cells. They used a genetic fate mapping system comprised of a transgene encoding a conditional, tamoxifen-dependent Cre recombinase under the regulation of the cardiomyocyte-restricted myosin heavy chain promoter in conjunction with a ubiquitously-expressed reporter transgene. Transient exposure to tamoxifen resulted in the generation of adult mice which expressed green fluorescent protein (GFP) in terminally differentiated cardiomyocytes, and beta-galactosidase in new cardiomyocytes arising out of stem cells. The study demonstrated a progressive increase in beta-galactosidase expressing cardiomyocytes following MI. During normal aging up to one year, the percentage of GFP^+ cardiomyocytes remained unchanged, indicating that stem or precursor cells did not refresh uninjured cardiomyocytes at a significant rate during this period of time. By contrast, after MI or pressure overload, the percentage of GFP^+ cardiomyocytes decreased from 82.8% in heart tissue from sham-treated mice to 67.5% in areas bordering a myocardial infarction, 76.6% in areas away from a myocardial infarction, and 75.7% in hearts subjected to pressure overload, indicating that stem cells or precursor cells had refreshed the cardiomyocytes. Thus, the study inferred that CSCs might participate in the formation of new cardiomyocytes after injury but not during the aging process. Using double transgenic MerCreMer-ZEG mice, Chan et al. [21] also tracked the fate of adult cardiomyocytes by the expression of GFP specifically induced in cardiomyocytes. Upon experimental MI, a reduction in GFP expression in the myocardium was observed, indicating the refreshment of cardiomyocytes by endogenous stem or precursor cells. Malliaras et al. [22] conducted genetic fate mapping to mark resident myocytes in combination with long-term BrdU pulsing and studied the origins of postnatal cardiomyogenesis in the normal, infarcted and cell-treated adult mammalian heart. The study documented that myocyte replenishment occurs almost exclusively through proliferation of small mononucleated adult cardiomyocytes in the normal adult mouse heart, without any measurable contributions by endogenous progenitors. They reported an annual endogenous cardiomyocyte turnover of 1.3% (if we consider all cases of binucleation and polyploidization as instances where cell cycle is activated abortively) to 4% (if we consider all measured DNA synthesis as formation of new myocytes). The study however reported that after MI, new cardiomyocytes arise from both, progenitors as well as pre-existing cardiomyocytes and also transplantation of CDCs upregulate host cardiomyocyte cycling and recruitment of endogenous progenitors, while improving heart function and increasing viable myocardium.

Other school of thought proposes that cardiomyocytes are the progeny of resident CSCs, which control cell turnover physiologically and cardiac repair following injury. Uchida et al. [23] generated triple-transgenic mice based on the tet-cre system to identify descendants of cells that have expressed the stem cell marker Sca1. They found a significant and lasting contribution of Sca1-derived cells to cardiomyocytes during normal aging. Ischemic damage and pressure overload resulted in

increased differentiation of Sca1-derived cells to the different cell types present in the heart. The study presents an evidence of continuous replacement of myocardial cells by Sca1$^+$ CSCs. Using a model of myocardial injury with patent coronary circulation to test the spontaneous regenerative capacity of resident CSCs, in-situ labeling and genetic tracking the fate of c-kit$^+$ cardiac stem cells and the replacement of the CSCs by transplantation of genetically tagged CSCs, Ellison et al. [9] provided an evidence that the CSCs autonomously repair extensive cardiac diffuse damage, leading to complete cellular, anatomical and functional cardiac recovery. The study showed that if the eCSCs are ablated, myocardial regeneration and ventricular performance is debilitated causing heart failure unless they are replaced by exogenous CSCs.

Another group of researchers however believe that CSCs have a non-significant role in cardiomyocyte renewal even during injury. Senyo et al. [24] combined two different pulse-chase approaches—genetic fate-mapping with stable isotope labeling and Multi-isotope Imaging Mass Spectrometry. They showed that genesis of cardiomyocytes occurs at a low rate by division of pre-existing cardiomyocytes during normal aging, a process that increases by four-fold adjacent to areas of myocardial injury. The study concluded that cardiac progenitors do not play a significant role in myocardial homeostasis in mammals and suggests that their role after injury is also limited. Ali et al. [25] used several transgenic mouse models that enable clonal analysis of postnatal cardiomyogenesis. They provided a new line of evidence for the differentiated α-myosin heavy chain-expressing cardiomyocyte as the cell of origin of postnatal cardiomyogenesis using the "mosaic analysis with double markers" mouse model. The observations of the study also argue against the existence of robust putative stem cells. The capacity to divide postnatally appears to be restricted to a small fraction of cardiomyocytes, and this property diminishes over time and appears insensitive to stimulation by an infarction injury within a 4-week time period after the infarct [25]. In another recent study, Berlo et al. [26] generated mice in which the *Kit* locus was used for lineage tracing analysis to examine if and how frequently c-kit$^+$ cells generate cardiomyocytes in vivo. The study revealed that c-kit$^+$ cells have the ability to contribute to the cardiomyocyte compartment of the heart and loss of the *Kit* gene, which is known to compromise the progenitor and migration activity of c-kit$^+$ cells, completely prevent cardiomyocyte formation from c-kit$^+$ cells. However, throughout development, with aging or with cardiac injury, the percentage of cardiomyocytes emerging from the c-kit$^+$ lineage is very low and hence highly unlikely to significantly affect cardiac function [26].

Given the disparity of results regarding the role of CSCs in cardiomyocyte renewal, it may be argued that the endogenous or transplanted CSCs, even if not involved in direct cardiovascular differentiation, may contribute towards myocardial repair by secretion of paracrine factors. Moreover, it should be noted that most of the lineage tracing and radioactive thymidine labeling studies suffer from one or the other technical limitations such as inappropriate labeling of the cells, measurement of DNA synthesis and not actual cell division, toxicity attributed to thymidine labeling etc. Most importantly, lineage-tracing protocols cannot be performed in humans and hence results from animal studies should be cautiously extrapolated to human CSCs.

7.4 Animal Studies with CSCs

A plethora of studies have demonstrated that isolated and culture-expanded c-kit^{+}/Lin^{-} CSCs exhibit all the properties of bonafide stem cells, and when injected into the injured myocardium, are capable of restoring (to a variable extent) the cardiac structure and function in various animal models. Several parameters such as infarct size, left ventricular ejection fraction (LVEF), LV volumes, cardiac output, LV segmental wall thickening and ventricular remodeling have been assessed as critical end-points after CSC therapy. Studies indicate that the administration of CSCs can slow left ventricular remodeling and improve cardiac function in both acute and chronic models of MI. In the first report on CSCs, Beltrami et al. [1] injected 1×10^5 culture expanded ckit-pos cells into the hearts of syngeneic rats acutely after myocardial infarction (5 h old infarcts) and reported that CSCs regenerate more than 50% of the contractile myocytes and vascular cells normally present in the myocardium [1]. Dawn et al. [27] performed an intravascular delivery of rat CSCs in a clinically relevant rat model comprising of temporary coronary occlusion followed by reperfusion. CSCs induced myocardial regeneration and decreased infarct size by 29%. Further, the study indicated that cell fusion did not contribute to tissue reconstitution [27]. Bearzi et al. [28] injected human CSCs in the immunodeficient mouse or imunocompromised infracted rat heart to form chimeric organs containing human myocytes and coronary vessels. The hCSCs differentiated into human myocytes and coronary vessels, leading to the formation of a chimeric heart in the recipient animals. Further, the human myocardium structurally and functionally integrated with the rodent myocardium and contributed to the performance of the infarcted heart. This study also ruled out any possibility of cell fusion between human CSCs and rodent cells [28].

Tang et al. [29] infused GFP-tagged CSCs in rats one month after coronary occlusion/reperfusion injury via intracoronary route. At 5 weeks post-transplantation, CSC-treated hearts exhibited improvements in both LV structure and function, as demonstrated by greater viable myocardium in the risk region, less fibrosis in the non-infarcted region, and improved ejection fraction (EF) in comparison to the vehicle controls [29]. Bolli et al., demonstrated that an intracoronary administration of autologous CSCs to a swine model of chronic ischemic cardiomyopathy resulted in a significant increase in LV function, indicated by an increase in EF and systolic thickening fraction in the infarcted LV wall, as well as a decrease in LV end-diastolic pressure one month post-infusion. The study also demonstrated using GFP-labeled CSCs that newly cardiomyocytes and vascular structures were derived from the transplanted cells [30]. In another similar study, Welt et al. [31] used a canine model of chronic infarction and late adverse ventricular remodeling and demonstrated that after six weeks of coronary ligation, intramyocardial injection of autologous CSCs resulted in significant improvement in LV volumes and LVEF compared with controls even at 30 weeks post-infarction, indicating that CSCs also have a beneficial effect on the late phase of cardiac remodeling in the chronically infarcted canine heart.

Studies by Marban's group have cultured human endomyocardial biopsies-derived CSCs into CDCs and transplanted these into acute myocardial infarcts in immunodeficient mice and pig model of heart failure after myocardial infarction [16, 32]. The results depict that the transplanted cells engraft and migrate into the infarct zone and lead to improvement in cardiac functions such as LVEF and attenuation of ventricular remodeling. In another study by Suzuki G, it has been demonstrated that slow infusion of CDCs into the three major coronary arteries (total dose: 30 million CDCs) in swine with hibernating myocardium improved regional function in ischemic LAD as well as in the normal right coronary artery regions (68–107%, $P < 0.05$) and ejection fraction [33].

In a study by Lee et al., the investigators have compared the effects of CDCs and their precursor cells, cardiospheres, which are heterogenous groups of cells that contain not only adult CSCs, capable of long-term self-renewal and cardiomyocyte differentiation, but also vascular cells and differentiated progenitor cells in a swine MI model [32]. The study has reported that the effects on infarct reduction and preservation of EF is similar in both CDCs and cardiospheres, however hemodynamics, regional function and preservation of LV chamber remodeling are improved in animals receiving cardiospheres, suggesting the benefits of heterogeneous cell therapy.

7.5 Clinical Trials with CSCs

Although there are many success stories of CSCs in experimental models, many challenges still await their therapeutic use in the clinical arena. Encouraging results from the animal studies formed the basis for the first clinical trial of c-kit+ CSCs, Cardiac Stem Cell Infusion in Patients with Ischemic Cardiomyopathy (SCIPIO). SCIPIO involved 23 patients who had experienced MI in the past and exhibited an EF of under 40%. One million of autologous $cKit^+$ and lineage negative CSCs were isolated with magnetic beads from cultures of right atrial appendage tissue and administered via intracoronary infusion 1 month after coronary artery bypass grafting (CABG). Twelve months after the treatment, infarct size was decreased by 30.2%, regional wall thickening was increased by 18% and left ventricular EF was increased by 8.2%. The benefits of treatment continued to increase and left ventricular EF was increased by 12% after 2 years [34].

Another trial, named CADUCEUS, which is CArdiosphere-Derived aUtologous stem CElls to reverse ventricULar dySfunction trial evaluated the safety and efficacy of intracoronary autologous CDCs in 17 patients with left ventricular dysfunction after MI. Six patients were included as control patients who were not given CDC treatment. The cardiospheres were expanded ~36 days in culture from right ventricular endomyocardial biopsies taken 2–4 weeks after acute myocardial infarction and injected into the previously stented coronary artery between 6–12 weeks after the heart attack. Despite the lack of improvement in left ventricular EF or patient reported outcomes, there were reductions of scar mass in CDC-treated patients by

28% and 46% at 6 and 12 months respectively. Also, no complications were reported within 6 months of CDC infusion. Further, a 1 year follow up of the CADUCEUS trial patients was performed [35]. MRI revealed that CDC-treated patients had smaller scar size compared with control patients and the scar mass decreased and viable mass increased in CDC-treated patients but not in control patients. Also, the changes in left ventricular ejection fraction in CDC-treated subjects were consistent with the natural relationship between scar size and ejection fraction post-MI.

Since autologous therapy is associated with significant technical, timing, economic and logistic limitations, researchers have now started exploring the potential of allogeneic CDC therapy. CDCs exhibit a favorable immunologic antigenic profile and are hypoimmunogenic in vitro. Hence, allogeneic human CDCs are currently being tested clinically in the ALLSTAR and DYNAMIC trials [36]. Another small phase-1 trial, ALCADIA (AutoLogous Human CArdiac-Derived Stem Cell To Treat Ischemic cArdiomyopathy) administrated autologous CDCs together with a controlled release formulation of basic Fibroblast Growth Factor (bFGF) in six patients with ischemic cardiomyopathy and heart failure. At 6 months, four patients showed an increase in LVEF and infarct size decreased by 3.3% of the total LV volume and maximal aerobic exercise capacity increased. The results of the trial were however not published, owing to small sample size [37].

Although the initial results of the trials are promising, CSC therapy is still in its infancy. This may be ascribed to various impediments, including, small numbers of CSCs that can be isolated from biopsies, limited numbers available for transplantation and poor survival and retention of the injected cells in the heart. Thus, increasing survival and retention of the transplanted CSCs in the heart currently constitutes one of the major challenges in the field of CSC therapy. In this regard, multiple protocols have been developed to optimize the survival and expansion of both ex-vivo transplanted and endogenous human c-kit-positive CSCs for clinical use.

7.6 Engineering of CSCs: Ex-Vivo Manipulation Studies

Since the isolation and identification of CSCs, multiple protocols have been developed to optimize the expansion of human c-kit-positive CSCs for therapeutic use (Fig. 7.1). Various groups have generated multicellular clusters known as cardiospheres, which have yielded cardiosphere-derived cells (CDCs) cardiospheres from human endomyocardial biopsy specimens. Human cardiospheres express c-Kit and CD105, a regulatory component of the transforming growth factor-β receptor complex important in angiogenesis and hematopoiesis [15, 16]. Cells within the cardiosphere core are proliferative, as identified by Ki67 expression. Human and porcine CDCs also differentiate into electrically functional myocytes in vitro and when injected into mice, these CDCs also lead to myocardial regeneration and functional improvement after infarction [16]. Gouman et al., have described efficient isolation and propagation of human cardiomyocyte progenitor cells (hCMPCs) from fetal heart and patient biopsies. Establishment of hCMPC cultures have been remarkably

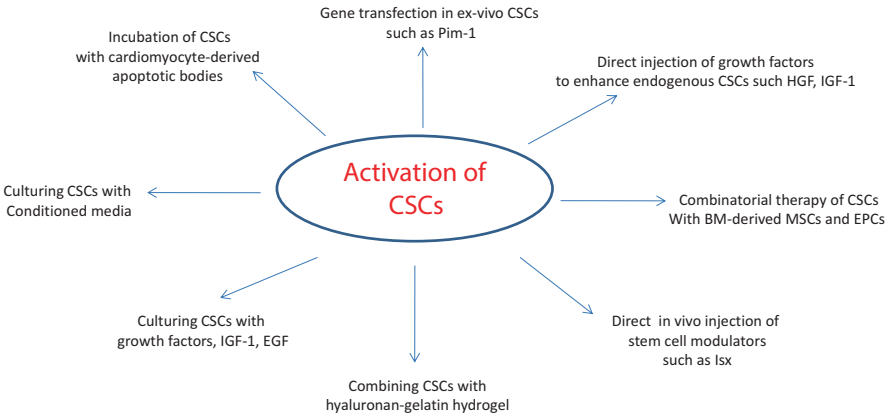

Fig. 7.1 Strategies used for the in vitro and in vivo activation of cardiac stem cells (CSCs)

reproducible, with over 70% of adult atrial biopsies resulting in robustly expanding cell populations. Following the addition of transforming growth factor beta, almost all cells have exhibited differentiation into spontaneously beating myocytes with characteristic cross striations [38]. Tang et al. [39] have described a fibroblast-free conditional CGM medium to expand Sca-1+ cells from small amount of heart tissue. The study showed that Sca-1+ cells keep their capacity for self-renewal and clonogenic in vitro with fibroblast-free conditional CGM medium, and can differentiate into cardiomyocytes, endothelial cells, and smooth muscle cells after being transplanted into ischemia-induced heart of mice. Excluding fibroblasts from CSC culture is essential because fibroblasts maintain high proliferative potential, and will overgrow CSCs in cardiospheres [39]. A study by our group has investigated the effects of different growth factors on CDCs. CSCs appear to respond to epidermal growth factor (EGF) more efficiently than other widely used growth factors such as vascular endothelial growth factor, insulin-like growth factor, basic fibroblast growth factor, hepatocyte growth factor, transforming growth factor, and platelet-derived growth factor. Pretreatment with EGF enhanced the expression of cardiac markers cTN1(+) and MHC(+) in CDCs in comparison to untreated controls [40]. Another study demonstrated that factors such as PDGF-AA present in MSC-CM improve migration of resident stem cells from human cardiac tissue [41].

Studies by Kawaguchi et al. [42] have demonstrated via in vitro co-culture experiments that c-kit^{+} CSCs with high expression of GATA-4 enhance the survival and contractility of adult cardiomyocytes through increased IGF-1 levels and induction of the IGF-1R signalling pathway, modulates the paracrine survival effect of c-kit^{pos} GATA-4 high CSCs on adult cardiomyocytes in vitro. The study has shown that CSCs which express high levels of GATA-4 have a pro-survival effect on cardiomyocytes due to up-regulation of IGF-1 [42]. c-kit^{+} CSCs are also known to express TNFR2 and that binding of TNF to this receptor can result in CSC activation and cell cycle entry in association with Lin-28 [43]. The actin monomer binding peptide, Thymosin β4 (Tβ4), has recently been described as a powerful regenerative

agent with angiogenic, anti-inflammatory and cardioprotective effects on the heart and which specifically acts on its resident cardiac progenitor cells. Tβ4 is known to activate the quiescent adult epicardium and specific subsets of epicardial progenitor cells for repair [44]. Recently, Tyukavin et al. [45] reported that the addition of cardiomyocyte-derived apoptotic bodies to the culture of neonatal myocardial cells stimulated proliferation and differentiation of cardiomyocyte precursors and the frequency of their contraction was 1.5-fold higher than in the control. Also, systemic administration of cardiomyocyte-derived apoptotic bodies to Wistar rats with chronic postinfarction heart failure during the early period of myocardial remodeling considerably improved the contractile function of the heart [45].

Several studies have tested different strategies to overcome the problem of poor survival or retention of the cells in the hostile environment of the infracted heart. Mohsin and colleagues tested the effect of ex vivo gene delivery of a pro-survival gene, Pim-1 kinase on survival/engraftment and reparative potential of human CSCs using a mouse model of ischemic cardiomyopathy [46]. Human CSCs engineered to overexpress Pim-1 were superior over the control cells in terms of cellular engraftment and differentiation. Also, Pim-1 overexpression does not lead to immortalization or oncogenic transformation of CSCs. To enhance retention and engraftment of CDCs, Cheng et al. [47] have conducted the intramyocardial injection of CDCs in a hyaluronan-gelatin hydrogel that improved retention, engraftment and efficacy in preclinical studies. The CDC-hydrogel combination therapy reduces cell loss due to leakage by virtue of hydrogel viscosity and by acting as a substrate to which CDCs can anchor and allows for the gradual migration of CDCs out of the hydrogel and show prolonged paracrine effects. Pharmacologic activation of innate cytoprotective mechanisms is also a lucrative option to enhance the in vivo survival and engraftment of CSCs. Cai et al. have shown that treatment of human c-kit+ CSCs with cobalt protoporphyrin (CoPP), a well known HO-1 inducer, promoted cell survival after increased oxidative stress in vitro [48]. The cytoprotective effects of CoPP are dependent on the upregulation of HO-1, cyclooxygenase-2, and nuclear factor-like 2. Interestingly, preconditioning CSCs with CoPP also lead to a global increase in release of a variety of cytokines, and the conditioned medium from cells pretreated with CoPP conferred naive CSCs remarkable resistance to apoptosis, demonstrating that cytokines released by preconditioned cells also play a major role in the pro-survival effects of CoPP [48].

7.7 Stimulation of Endogenous CSCs

CSCs produce a repertoire of pro-survival, anti-inflammatory and cardiovascular regenerative growth factors such as: IGF-1, HGF, TGF-β1 superfamily, including activins and BMPs, neuregulin-1, periostin, and BMP-10 among others [49]. It has been proposed that although the transplanted CSCs themselves survive only transiently and do not directly participate in the production of cells that contributes to the regenerated tissue, intracoronary injection of allogeneic CSCs in a clinically

relevant MI model activates the resident host CSCs resulting in improved myocardial cell survival, function, remodeling and regeneration. Thus, although the therapeutic cells are allogeneic, the regenerative response may be completely autologous because it is carried out by the resident CSCs. Henceforth, besides using expanded CSCs for cardiac repair, several studies have also attempted to deliver growth factors to stimulate resident CPCs and promote myocardial regeneration. Intramyocardial delivery of hepatocyte growth factor (HGF) and insulin-like growth factor-1 (IGF-1) have been employed because CSCs express c-Met and IGF-1-receptors and HGF is a powerful chemoattractant of CSCs while IGF-1 promotes their division and survival. Results have demonstrated that CSCs locally activated by HGF and IGF-1 directly in proximity of a healed infarct can salvage nearly 45% of the infarct by replacing fibrotic tissue with fully functional myocardium. Intracoronary administration of IGF-1 and HGF has been shown to affect the paracrine effects of endogenous CSCs [50]. A single dose ranging from 0.5 to 2 μg HGF and 2–8 μg IGF-1, administered just below the site of left anterior descendent occlusion, 30 min after acute myocardial infarction (AMI) during coronary reperfusion in the pig trigger a regenerative response from the c-kit$^+$ CSCs, which is potent, self-sustained and able to produce significant restoration of the damaged myocardium without the need for cell transplantation [50]. IGF-1 and HGF have also been reported to induce CSC migration, proliferation and functional cardiomyogenic and microvasculature differentiation. Furthermore, these growth factors, in a dose-dependent manner, have been shown to improve cardiomyocyte survival, reduce fibrosis and cardiomyocyte reactive hypertrophy [51]. Similar positive effects have been obtained when the HGF-IGF1 combination is administered trans-endocardially in pigs with a chronic MI using the NOGA system [52]. Paracrine engineering of explant-derived CSCs to overexpress IGF-1 has been depicted to substantially improve cardiac stem cell-mediated repair by enhancing the long-term survival of transplanted cells and surrounding myocardium [52]. Russell et al. 2015 have shown that 3,5-disubstituted isoxazoles (Isx), stem cell-modulator small-molecules originally recovered in a P19 embryonal carcinoma cell-based screen, directs muscle transcriptional programs in vivo in multipotent Notch-activated epicardium-derived CSCs, generating Notch-activated adult cardiomyocyte-like precursors. The study has documented that Isx, administered to adult mice as a once daily intra-peritoneal (ip) injection, robustly activate cardiac gene programs in multipotent CSCs in vivo, a promising start for a cardio-regenerative small-molecule. The cardiac regenerative effects of Isx in MI models, however still warrants further investigation [53].

7.8 Other Stem Cell Sources for Heart Regeneration

Besides cardiac stem cells, various cell types at different developmental stages, including adult cells, fetal and embryonic cells have been considered for transplantation into the heart. Initial cardiac cell transplantation efforts have been done using skeletal myoblasts (SMBs), adult stem cells isolated from skeletal muscle biopsies

[54]. Based on their utility in animal studies, SMBs have been utilized in several clinical trials in patients with severe left ventricular dysfunction post-infarction [55, 56]. Follow-up studies have shown a moderate, but significant increase in the LVEF. Fetal cardiomyocytes have also been used for cardiomyocyte regeneration in the 1990s [57–59]. These cells significantly improved cardiac functions and angiogenesis in the injured animals [59].

Three populations of stem cells in the bone marrow (BM), HSCs, MSCs and endothelial progenitor cells (EPCs) have been reported to contribute to heart muscle repair. The ability of transplanted bone marrow (BM)-derived HSCs to regenerate the infarcted myocardium has been first shown in 2001 [60]. All functional HSCs are Lin^- and display high levels of Sca1 and c-kit. The study demonstrated that $ckit^+$ HSCs trans-differentiated into mature cardiomyocytes, smooth muscle cells, and endothelial cells in a murine model of MI and resulted in improvement of LVEF in the infracted heart [60]. Although subsequent studies have challenged the transdifferentiation of HSCs into heart muscle cells, the therapeutic efficacy of BM-HSCs have been proven in many studies [61]. Apart from c-kit, many other cell surface markers have also been identified that define populations enriched for freshly isolated human HSCs, including the CD133+ and CD34+ hematopoietic cells. MSCs represent less than 0.1% of the BM-mononuclear cells and can be identified as a subset of cells expressing Sca 1. MSCs have been shown to differentiate into cardiomyocytes as well as vascular endothelial cells in vitro. However, experimental evidence suggests that when transplanted in vivo, MSCs contribute to neo-vascularisation and cardiomyocyte protection, via the secretion of paracrine factors. A study has reported that combined transplantation of hCSCs and hMSCs into the infarct border zones at 14 days after MI in a swine model leads to twofold-greater reduction in scar size compared with either cell administered alone and also restores diastolic and systolic function toward normal after MI [62]. Major advantages of using MSCs are firstly they can be isolated from a variety of tissues, including bone marrow, adipose tissue, cord blood, and also can also be substantially expanded in vitro. Secondly, they lack major histocompatibility complex II and B7 co-stimulatory molecule expression, which makes them tolerogenic in the host and thus can be given allogenically. EPC act as major players in marrow angiogenesis due to their relevant clonogenic potential. EPC have been identified by cell surface markers including CD34, CD133 and vegfr2. EPC isolated from peripheral blood and/or BM has shown incorporation into sites of physiological and pathological neovascularization in the endothelium after either systemic injection or direct intramyocardial transplantation in animal models of peripheral limb ischemia and myocardial infarction [63–65]. *Ex vivo* expanded gene-modified EPC have been reported to enhance EPC proliferation, adhesion and impaired neovascularization in an animal model of experimentally induced limb and myocardial ischemia [66, 67]. In our studies, we have demonstrated that EPCs modified with endothelial nitric oxide synthase gene show enhanced proliferation, migration and neovascularization both in vitro and in vivo in rabbit model of hind limb ischemia [68, 69].

Various clinical trials have been conducted using BM-stem cells including the BOOST trial, REPAIR-AMI Trial. Results have demonstrated improvement in

LVEF at 4 months and reduction in combined clinical end points of death, recurrence of AMI, and any revascularization procedure at 1 year. However, other groups from Belgium and Norway, have been unable to detect a difference in outcome between bone marrow cell treated group and controls in AMI setting [70]. Different cell isolation protocols as well as dosage, degree of cell viability and function prior to delivery may contribute to the heterogeneous clinical results in randomized trials. In the (transplantation of progenitor cells and recovery of LV function in patients with chronic ischemic heart disease) TOPCARE-CHD trial, the absolute change in LVEF at 3 months, was significantly greater among patients receiving the bone marrow cells than among those receiving circulating progenitor cells [71]. Cochrane Heart Group have studied 33 clinical trials (1765 patients) for effectiveness of BM-cells for cardiac regeneration following acute MI. They have concluded that while no significant improvement was observed in the mortality and morbidity of the patients who received BM-cells, a significant and sustained improvement (in LVEF was there during 12–61 months follow-up period [72]. In another meta-analysis, the same group has reported that, in addition to the improvement in LVEF, BM-cells are also able to improve the morbidity and mortality in patients with chronic heart disease and congestive heart failure [73]. Although, much advancement has been made in the area of BM-stem cells therapeutics and cardiac regeneration, which and how specific population of cells from the BM actually contributes to cardiac repair is not yet conspicuous. Whatever it may be, it is definite that the BM-heart axis plays a pivotal role in heart regeneration after injury.

Exciting new advances in cardiomyocyte regeneration are also being made in human embryonic stem cell research. Studies have shown that hESCs can reproducibly differentiate in culture into embryoid bodies and the cells have structural and functional properties of early stage cardiomyocytes [74, 75]. In experimental studies, the transplantation of mESC-derived cardiomyocytes into the injured hearts of immunocompatible mice has resulted in the formation of stable intracardiac grafts [76]. In 2004, Kehat et al. reported human cardiomyocyte transplantation into the uninjured swine myocardium [77]. The transplantation of ESC-derived cardiomyocytes into normal and injured heart in animals has been shown to improve the global myocardial function, although for a short period of time. Efforts are now directed at identifying defined factors to enhance the differentiation of cardiomyocytes from hESC [78]. Recently Chong et al have succeeded in generating cardiomyocytes from ESCs on a large scale. These ESC-CMs are able to successfully engraft and repair the injured myocardium in a primate model of myocardial infarction [79]. Despite the evidence of ESCs efficacy in larger animal models, their clinical use has been hampered due to many reasons, including their genetic instability, risk of arrhythmias, potential tumorigenic and immunogenic properties, little improvement in cardiac functions and finally ethical considerations related to the origin of these cells. Besides these cell types, induced pluripotent cells (iPS) have also been converted to cardiac progenitors in vitro and upon intramyocardial delivery into adult infarcted animal hearts, these cardiogenic iPS progeny have shown proper engraftement without disrupting the host tissues [80–82]. Importantly, iPS-based transplantation have shown to restore post-ischemic cardiac performance with evidence of

increased left ventricular thickness, and improve electrical stability following *in situ* regeneration of cardiac, smooth muscle, and endothelial tissue throughout the 4-week follow-up period [83].

7.9 Conclusion

Given their lineage commitment to cardiac cell phenotype, undoubtedly, CSCs constitute a powerful form of therapy for cardiac repair. However, there is definitely something in the niche of heart that hinders the differentiation of cardiac precursors into new functional cardiomyocytes. Thus, an identification of these factors is requisite to stimulate both endogenous cardiac repair and in vitro expansion of CSCs that can be subsequently used for transplantation studies. Long-term objectives of CSC therapy include optimization of dosages and route of administration, improved survival cell survival and engraftment with electromechanical integration into the heart, hampering of adverse myocardial remodeling and significant improvement in contractility of the diseased heart. Moreover, it is also worthwhile to further investigate the role and underlying mechanisms of endogenous c-kit$^+$ CSC-mediated in physiological cardiac repair. An amalgamation of basic and clinical studies would be critical to reap the fruits from CSC-mediated therapy on clinical platforms. The targets are challenging and strenuous but not impossible and absolutely worthwhile.

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Chapter 8
Cardiac Imaging and Stem Cell Transplantation

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8.1 Stem Cell Therapy and Homing

Mesenchymal Stem Cells (MSCs) are non-hematopoietic stromal cells that can be isolated from other cells in marrow by their tendency to adhere to tissue culture plastic. The cells have many of the characteristics of stem cells for tissues that can roughly be defined as mesenchymal, because they can be differentiated in culture into osteoblasts, chondrocytes, adipocytes, and even myoblasts [1]. MSCs are rare in bone marrow, representing ~1 in 10,000 nucleated cells with the ability to expand manifold in culture while retaining their growth and multi-lineage potential. The presence of non-hematopoietic stem cells in bone marrow was first suggested by the observations of the German pathologist Cohnheim 130 years ago raising the possibility that bone marrow may be the source of fibroblasts that deposit collagen fibers as part of the normal process of wound repair [2].

His observations were extended by other groups, which later established that these cells were multipotent [3, 4]. MSCs and MSC-like cells have now been isolated from various sites other than the bone marrow, including adipose tissue, amniotic fluid, periosteum, and fetal tissues, and show phenotypic heterogeneity [5–8]. The in vitro characteristics of MSCs are identified by the expression of many molecules including CD105 (SH2) and CD73 (SH3/4) and are negative for the hematopoietic markers

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CD34, CD45, and CD14. From immunological standpoint MSCs (widely described as MHC I^+, MHC II^-, $CD40^-$, $CD80^-$, $CD86^-$) are regarded as nonimmunogenic and, therefore, transplantation into an allogeneic host may not require immunosuppression. MHC class I may activate T cells, but because of absence of costimulatory molecules, a secondary signal would not engage, leaving the T cells anergic [9].

It has been shown that MSCs, when transplanted systemically, are able to migrate to sites of injury suggesting that MSCs possess migratory capacity. These properties of MSCs make them ideal candidates for tissue engineering [10]. The in vivo behavior of MSCs is less well known as compared to the in vitro characterization of these cells. Prior studies have either performed site directed or systemic administration of cells. For example, repair of infarcted myocardium has been studied in multiple studies using bone marrow cells [11–13]. Injury to a target organ is sensed by distant stem cells, which migrate to the site of damage and undergo alternate stem cell differentiation [14]; these events promote structural and functional repair [15]. Animal models have conclusively shown that transplantation of BMCs induces angiogenesis. BMCs differentiate into cardiac-like muscle cells in culture and in vivo in ventricular scar tissue and improve myocardial function [16].

The mechanism by which MSCs home to tissues is not yet fully understood, but it is likely that injured tissue expresses specific receptors or ligands to facilitate trafficking, adhesion, and infiltration of MSCs to the site of injury. Given considerable potential for myocardial repair using stem cells, it is pertinent to use appropriate imaging techniques to monitor myocardial homing and biodistribution of these cells after therapeutic application in patients.

8.2 Cardiovascular Applications of Stem Cells

Cardiovascular disease remains the number one cause of morbidity and mortality in the United States and Europe. Over the past decade or so, several animal studies and clinical trials have supported the use of stem cells as a potential therapeutic modality in patients with acute myocardial infarction and end-stage congestive heart failure. Several different types of cells have been used in both animal and human studies to promote repair of the damaged myocardium. For cardiovascular applications, adult and embryonic stem (ES) cells are the two main sources. These can be delivered either through transvascular route or direct injection into the left ventricular wall. The goal is to deliver enough cells at the site of injury to maximize restoration of cardiac function [17, 20]. Injecting stem cells in the setting of myocardial infarction can promote cardiomyocyte formation with improvement in systolic function [18]. Improvement in systolic function has also been shown in cardiomyopathies as seen on cardiac MRI imaging following autologous bone marrow transplantation suggesting homing of stem cells in the injured myocardium [19–21].

A study conducted at the authors' institution used cardiac amyloidosis as a model for infiltrative cardiac disease to study outcome of stem cell therapy [19]. In cases of amyloidosis secondary to multiple myeloma, the underlying plasma cell dyscrasia

must be controlled to allow for the regression of tissue amyloid deposits. Hematopoietic cell transplantation permits delivery of myeloablative doses of melphalan and is an effective therapy for eligible patients to achieve hematologic response. However, whether such elimination of the plasma cell clone can lead to reversal of organ damage, such as cardiac amyloidosis, is not clear. The authors examined the possibility that autologous BMT might improve cardiac function in patients with MM and cardiac amyloidosis using cardiac MRI as the diagnostic tool. Our study showed significant improvement in LV systolic function in each of the patients with low ejection fraction following BMT. Interestingly, cardiac MRI showed no change in delayed enhancement pattern in these patients. This cardiac MRI pattern suggests that the improvement in LV systolic function was not due to resolution of amyloid infiltrate, but most likely it was related to bone marrow stem cells homing into the myocardium and differentiating into new cardiomyocytes that would participate in cardiac contractility. Some previous studies have indeed shown that homing of stem cells in the ischemic myocardium improves LV function and outcome [20, 21]. Experimental studies suggest that transfer of stem cells and progenitor cells can have a favorable impact on perfusion and contractile performance of the failing heart. Preliminary efficacy data indicate that stem cells have the potential to enhance myocardial perfusion and/or contractile performance in patients with acute myocardial infarction, advanced coronary artery disease, and chronic heart failure [20]. The intracoronary delivery of unselected bone marrow cells has recently been shown to enhance LV EF recovery in patients after acute myocardial infarction [21]. Our study suggests that some degree of heart failure may be a pre-requisite for improvement in cardiac function. The failing heart potentially creates a more compatible nidus for the homing of progenitor cells with release of many inflammatory markers and chemokines. The failing heart is also likely more apt for signaling and recruitment of bone marrow stromal cells to the myocardium following BMT. Notably, we were not able to show a direct correlation of a number of inflammatory signals such as CD-19, IL-6 and CD-4 and hs-CRP and improvement in LV function. There can be several reasons for lack of this correlation: these markers may not reflect chemokines and cytokines that induce mobilization and homing of progenitor cells; or measurement at a single time point before BMT may not correlate with improvement several months following BMT. Most likely, it is a combination of several known and unknown signals that induces mobilization, homing and transformation of stem cells into cardiomyocytes.

Similar to our observations, several other individual studies have shown improvement in cardiac function following stem cell therapy [18, 20–22]. However, conflicting results have also been noted in several multicenter phase 3 studies employing BMMC injection with no significant changes in ejection fraction [23, 24]. The variable results are likely due to the fact that successful regeneration in the heart requires the injected cell to be delivered to the correct zone of the myocardium, survive in the host microenvironment, exert beneficial paracrine effects, differentiate, and integrate with the host myocardium. Thus there are multiple variable that affect homing of adequate number of cells in the appropriate site and their cellular transformation into cardiomyoctes. The rapid washout of the stem cells from the myocardium is

possibly a major concern regarding the delivery of adequate number of stem cells to the injured myocardium with only a small fraction of stem cells remaining within the heart after injection [25]. Considering variable cardiac outcomes it is imperative that better methods of tracking stem cells and imaging are developed to determine the fate of these cells after transplantation.

8.3 Cardiac Imaging in Stem Cell Therapy

Imaging modalities that have been validated for stem cells tracking include fluorescence imaging (FI), bioluminescence imaging (BLI), positron emission tomography (PET), single-photon emission computed tomography (SPECT), magnetic resonance imaging (MRI) and computed tomography (CT) (Table 8.1). Each of these techniques has its own strengths and weaknesses with respect to the use in animal and human studies. BLI has been the most popular imaging modality for small animal studies [26] while planar FI has been limited to proof-of-principle studies [27]. Imaging modalities such as PET, SPECT, and MRI allow tomographic assessment of cells in both animals as well as humans. PET and SPECT, when combined with CT, have been particularly useful in quantifying the whole-body distribution of cells after delivery, whereas MRI has seen more utility in determining the transmural location of stem cells due to its superb spatial resolution [26, 28–32].

8.4 Non-invasive Methods of Cardiac Imaging Post Stem Cell Therapy

The ideal cardiac imaging modality should provide integrated information related to the entire process of cell engraftment, survival, and functional outcome following stem cell therapy. Established parameters of noninvasive imaging, such as contractile function, perfusion, and viability of the myocardium, do not provide direct visualization of transplanted cells, their biology or function [33] leading to use of contrast agents and detectors for noninvasive visualization of therapeutic cells in vivo. Ideal imaging technique for stem cell tracking should be biocompatible and safe with no genetic modification or perturbation to the stem cell. Techniques should allow cell quantification at any anatomic location with minimal or no dilution with cell division. Ideally these techniques should cause minimal or no transfer of contrast agent to non-stem cells [34]. Such imaging approaches may help understand the logistics in preclinical studies and may also have direct clinical applications.

Multiple imaging techniques have been used for in vivo imaging of labeled transplanted cells. Magnetic resonance imaging (MRI) uses super-paramagnetic iron oxide (SPIO) while radionuclide technology involves agents like In-111 oxin, F18-FDG and Tc-99m HMPAO to meet the broad objectives of stem cell tracking. MRI

Table 8.1 Functional effect of stem cell therapy and imaging techniques used

Study	Setting	Route of delivery	LVEF/technique used	Myocardial perfusion/technique used	Infarct size/ technique used	Myocardial viability/technique used
TOPCARE-AMI	AMI	Intracoronary	> (9.3%)/LV angiography, cine MRI	Decreased perfusion defect/TI-SPECT	Decreased/CMR	Increased F18-FDG around infarction/ F18-FDG PET
BOOST	AMI	Intracoronary	No change/cine MRI	NA	No change/CMR	NA
Chen et al.	AMI	Intracoronary	>(18.0%)/LV angiography	NA	Decreased/LV angiography	Increased F18-FDG around infarction/ F18-FDG PET
Janssens et al.	AMI	Intracoronary	No change/cine MRI	No change in perfusion defect/PET	Decreased/CMR	No change/ PET
Bartunek et al	AMI	Intracoronary	>(7%)/LV angiography	Decreased perfusion defect/Tc-SPECT	NA	Increased F18-FDG around infarction/ F18-FDG PET
ASTAMI	AMI	Intracoronary	No change/gated SPECT, MRI, ECHO	NA	No change/CMR	NA
TOPCARE-CHD	CMI	Intracoronary	> (2.9%)/LV angiography	NA	No change/CMR	NA
IACT	CMI	Intracoronary	>(8.0%)/LV angiography	Decreased perfusion defect/Tc-SPECT	Decreased/LV angiography	Increased F18-FDG around infarction/ F18-FDG PET
Fuchs et al.	AP	Intramyocardial	No change/echo	Increased stress perfusion/TI-SPECT	NA	NA
Beeres et al.	AP	Intramyocardial	>(4.0%)/gated SPECT, cine MRI	Decreased extent of ischemial/Tc SPECT	No change/CMR	No change in viability/F18-FDG SPECT
Perin et al.	HF	Intramyocardial	No change/LV angiography, Echo	No change in perfusion defect/Tc SPECT	NA	Improvement in injected segments/ electromechanical mapping

(continued)

Table 8.1 (continued)

Study	Setting	Route of delivery	LVEF/technique used	Myocardial perfusion/technique used	Infarct size/ technique used	Myocardial viability/technique used
Strauer et al.	AMI	Intracoronary	No change/LV angiography	Decreased perfusion defect/TI-SPECT	Decreased/LV angiography	No change in contractile reserve/DSE
Katritsis et al.	CMI	Intracoronary	No change/echo	Decreased perfusion defect/Tc-SPECT	Decreased/Tc SPECT	Increased viability/DSE

AMI acute myocardial infarction, *CMI* chronic myocardial infarction, *AP* angina pectoris, *HF* heart failure, *LV* left ventricular, *NA* not available, *MRI* magnetic resonance imaging, *DSE* dobutamine stress echocardiography, *F18-FDG* F18-fluorodeoxyglucose, *PET* positron emission tomography, *Tc* technetium, *Tl* thallium, *SPECT* single-photon emission computed tomography; Studies: *TOPCARE-AMI* transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction, *ASTAMI* evaluation of treatment with intracoronary mononuclear autologous bone marrow cell therapy among patients with acute anterior myocardial infarction treated with primary percutaneous coronary intervention, *BOOST* bone marrow transfer to enhance ST-elevation infarct regeneration, *IACT* regeneration of human infarcted heart muscle by intracoronary autologous bone marrow cell transplantation in chronic coronary artery disease, *MAGIC* effects of intracoronary infusion of peripheral blood stem cells mobilized with granulocyte colony-stimulating factor on LV systolic function and restenosis after coronary stenting in MI, *MRI* magnetic resonance imaging, *TOPCARE-CHD* transplantation of progenitor cells and regeneration enhancement in congenital heart disease

Table 8.2 MRI versus nuclear techniques for in vivo imaging of labeled transplanted cells

Method	Advantages	Disadvantages
MRI	No radiation with high resolution.	Low sensitivity. Possible non-reflection of viable cells.
Nuclear (direct labeling)	High sensitivity with high translational capacity.	Radiation exposure to individual and therapeutic cells.
		Decay of radioactivity, not reflection of viable cells.
Nuclear (reporter genes)	High biologic specificity.	Limitations due to weak signal and potential adverse effect of gene modification.

has the advantage of higher resolution and no radiation but comes with issues of low sensitivity while nuclear imaging provides higher sensitivity with a disadvantage of radiation exposure (Table 8.2). Other techniques that have been introduced including genetic labeling with reporter genes that can be traced with imaging probes allowing for repeatable tracking of cellular and subcellular function over a longer period of time.

8.4.1 Direct Labeling of Cells Using Magnetic Resonance Agents

MRI has become a fast and comprehensive technique for the assessment of cardiac volumes, function, and mass in HF that is accurate but also highly reproducible [35]. Tracking transplanted stem cells using this technique with refined contrast agents offers biologic insight into homing and engraftment. Contrast agents including micron-scale particles like iron oxide and iron fluorophore particle (IFP) ensure maximum signal with minimum labeling. These agents thus provide detection of single cells at a resolution that can be achieved in vivo while the cells retain biologic activity with preservation of colony-forming ability and differentiation capacity [36, 37]. Similar results can be achieved by nanoparticles of iron oxide for non-toxic labeling of hematopoietic bone marrow-derived and mesenchymal stem cell populations without affecting their transdifferentiation capacity [38]. Direct delivery can be coupled with cell labeling in cardiac stem cell transplantation during endomyocardial injections. These labeled transplanted cells could be imaged shortly after delivery with a high degree of spatial resolution using MRI [39]. The lowest detectable number of cells is around 10^5 with use of conventional MRI scanners without any sequence modification. This can be lowered using high-field magnets such that single cells containing a single iron particle can be detected and tracked [40]. The disadvantage for MRI include inability of imaging signal to link with viability. Also, there is a possible risk of accumulation of magnetic resonance agents after cell death into surrounding cells causing incorrect assessment of cell trafficking. Newer direct labeling techniques like

Clio-tat peptides [41] or magnetic relaxation switches [42] have the ability to track the distribution and differentiation of progenitor and stem cells by high-resolution in vivo imaging techniques having significant clinical and research implications.

8.4.2 Direct Labeling of Cells Using Radionuclides

Direct labeling with radionuclides like Indium (In)-111 provides lot of valuable information in stem cell homing. Current data suggests that a small number of cells ultimately home to injured myocardium while a significant proportion of cells accumulate in other organs like lungs, this at the same time corroborates with high sensitivity of nuclear imaging technique [43, 44]. Homing of In-111–labeled stem cells to infarcted myocardium has been successfully visualized using imaging techniques like single-photon emission computed tomography (SPECT)-CT [39]. Other modalities like 18F-FDG and 3D PET scanning have been used following therapeutic application of intracoronary autologous bone marrow cell (BMCs) transplantation in patients with acute myocardial infarction. Following site directed and systemic administration the unselected BMCs labeled with 18F-FDG can be detected in the infarcted myocardium while the remaining activity can be found other organs like liver and spleen [21]. Unfortunately, because of the short half-life of ^{18}F-fluorodeoxyglucose, other isotopes with a longer half-life may need to be evaluated for optimal long-term tracking of stem cells. Also, use of intravenous route causes lesser degree of engrafting of stem cells as compared to site directed delivery of stem cells. It is well known that early infusion results in significantly higher uptake in the heart [29]. Accumulation of injected stem cells can be seen within hours after intracoronary infusion and outside of the myocardium higher stem cell accumulation is seen in spleen, liver, bladder and bone marrow. The delayed images on PET scan show a prolonged residence of stem cells at the myocardium [29]. Use of agents like technetium (Tc)-99m exametazime (HMPAO) has demonstrated the dynamic nature of cardiac cell engraftment after trans coronary transplantation in patients with acute myocardial infarction [45]. In comparison to MRI, radionuclide techniques have the advantage of a lower background signal and higher sensitivity at the cost of lower spatial resolution.

8.4.3 Reporter Genes for Cardiovascular Cell Imaging

Imaging reporter gene expression is a useful technique for noninvasive monitoring of gene therapy [46]. These reporter genes can be transferred to cells for genetic labeling prior to in vivo administration. These can later be detected by radio-labeled or optical reporter probes specific for the reporter gene within the transduced cell. Use of reporter gene labeling is more specific and requires the expression of the reporter gene and activity of the reporter-gene product which in turn depends on viability of therapeutic cells [47].

Even though the reporter-gene imaging is still mostly limited to animal model studies the genetic labeling holds promise towards deciphering subcellular mechanisms that take place within therapeutic cells. Also, further studies need to be done to establish an approach that is practical and can be used within clinical setting.

8.5 Cardiac Imaging for Functional Effects of Stem Cell Therapy

Cardiac Imaging can be used to evaluate the functional changes following stem cell therapy. This mainly includes changes in left ventricular (LV) function, myocardial perfusion, infarct size and myocardial viability. A summary of various imaging techniques used to study the functional effect of stem cell therapy is provided in Table 8.1.

8.5.1 LV Function

Multiple studies have suggested changes in LV function following stem cell therapy. This includes studies in patients with acute myocardial infarction and chronic ischemic heart disease. Although the results for some studies remained inconclusive [48, 49] several studies including work at the authors' institution suggest some improvement in LV function post stem-cell therapy [19, 50–52]. While multiple imaging modalities have been employed for evaluation of LV function, including LV angiography, 2-dimensional echocardiography, gated SPECT and MRI, gated SPECT and MRI are probably the most accurate modalities for assessment of LVEF and LV volumes. The global improvement of LVEF in these studies was mainly related to an improvement of regional LV function in the infarct zone, although improvement along the infarction border zone has also been reported. For most studies left ventricular end diastolic volume did not change suggesting absence of reverse modeling. A variation in time course of LV function was also noted in BOOST study [53] which showed that the improvement in LV function did not persist for a longer duration of time and beyond 18 months there was no difference when compared to the control group.

8.5.2 Infarct Size

Various cardiac imaging modalities have been used post stem cell transplant to evaluate the infarct size [51, 53, 54, 56]. These studies include both patients with acute myocardial infarction as well as chronic ischemic heart disease. Most studies evaluated the infarct size between 3 and 18 months time period using either direct

visualization of the infarct size (i.e., SPECT, contrast-enhanced MRI) or indirect method of measuring systolic dysfunction in the infarct zone as an indicator of extent of scar tissue. Though certain studies showed decrease in the infarct size over period of time [50–52] the results of the other studies was not conclusive [49, 56]. As far as imaging techniques is concerned contrast enhanced MRI provides more accurate results as compared to SPECT. MRI and SPECT detect transmural myocardial infarcts at similar rates. However, MRI systematically detects subendocardial infarcts that are missed by SPECT [57]. Considering variable results further randomized controlled trials are needed to evaluate changes in infarct size after stem cell therapy.

8.5.3 Myocardial Perfusion

Studies have used cardiac imaging tools to assess myocardial perfusion following stem cell therapy [51, 54, 55]. Changes in perfusion following acute myocardial infarction as well as chronic ischemic disease can be evaluated. These techniques mostly include nuclear imaging with PET or SPECT. While SPECT is predominantly used non-invasive imaging and provides information on relative changes in tracer uptake, PET measures absolute quantification of myocardial perfusion. Doppler flow wire can be used invasively to assess coronary blood flow at rest and stress [58]. Studies have shown improvement in perfusion defect following stem cell therapy with decrease in size of the defect seen over 3–12 months using resting Tc-99m sestamibi SPECT [59]. Also some studies have reported decrease in stress-inducible ischemia [60] in patients with refractory angina.

8.5.4 Myocardial Viability

Myocardial viability can be evaluated using nuclear imaging with PET (mainly using F18-FDG) or SPECT (with F18-FDG or Tc-99m-labeled agents), or low-dose dobutamine echocardiography or MRI. These techniques can be used to evaluate viability in the infarct zone with increased F18-FDG seen after cell therapy [51, 61] within 3–6 month of follow up. Also catheter-based electromechanical mapping can be used for identification and localization of viable myocardial tissue. Other marker of myocardial viability is contractile reserve with most studies not being able to show significant improvement following stem cell therapy. This may be because in patients with severe myocardial dysfunction and injury on the cellular level, contractile reserve is frequently lost, whereas glucose utilization is preserved. The substantial number of myocardial segments with preserved glucose and fatty acid utilization but without contractile reserve, suggests an underestimation of myocardial viability by dobutamine echocardiography [62]. Further studies would be needed to evaluate changes in myocardial viability following stem cell therapy.

8.6 Summary

Stem cell therapy in cardiac patients seems promising while many issues remain unanswered. There is a growing knowledge base of imaging techniques being used both in preclinical and clinical settings. Direct tracking of stem cells can be performed using radionuclides and super-paramagnetic agents while functional assessment can be performed using MRI, nuclear imaging with PET and SPECT, and echocardiography. Multiple studies have shown some degree of LV function and myocardial perfusion/viability with a reduction in infarct size while the results from other studies remains inconclusive. Further randomized, controlled trials with concurrent imaging techniques are needed to confirm and determine beneficial effects of cell-based therapies.

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Chapter 9
Bone Marrow Cell Therapy for Ischemic Heart Disease and the Role of Cardiac Imaging in Evaluation of Outcomes

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Cardiac imaging, in its many forms, is an indispensable tool for clinicians and scientists to assess the structure and function of the heart, both in physiological and pathological states. Over the recent years, 2-dimensional (2D) and 3D echocardiography, left ventriculography (LVG), computed tomography (CT), magnetic resonance imaging (MRI), single-photon emission computed tomography (SPECT), and positron emission tomography (PET) have transitioned from being concept techniques to integral components of cardiovascular healthcare delivery. Today, cardiac imaging plays a major role in everyday practice, in outpatient clinics as well as inpatient services. Imaging modalities are now ubiquitous; simpler versions like transthoracic 2D echocardiography (TTE) equipment or handheld machines are found virtually everywhere, while more sophisticated gadgets like cardiac MRI and SPECT are found in most large hospitals. The relatively easy availability of these powerful devices has transformed modern day cardiac care.

Along with these advances in imaging, newer options with medical therapy in recent years have helped reduce the impact of an acute myocardial infarction (MI), with marked improvement in patient outcomes. With better access to healthcare, patients are being transported to an ER and provided superior care, faster than ever before [1]. This enables physicians to minimize the damage from the acute event, and thereby improve long-term outcomes for the patient. Over the past three decades, patients with ischemic heart disease have also benefitted tremendously from the discovery of revolutionary drugs, such as β-blockers, angiotensin converting enzyme inhibitors, and statins. These drugs have had profound impact on

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outcomes of ischemic heart disease patients by reducing morbidity and mortality [2, 3]. However, despite their manifold benefits, these agents are incapable of replacing myocardial tissue that is lost due to ischemic injury. Therefore, there remains a need for newer forms of therapy, which may potentially reverse the loss of functional cardiomyocytes and induce effective myocardial reconstitution. Since the turn of the century, stem cell therapy has emerged as a viable candidate to fill this therapeutic void. By virtue of their reparative capabilities, cell therapy may indeed potentially heal cardiac tissue that was once considered permanently lost.

Because of this unprecedented promise of cardiac repair potentially attainable with cell therapy, numerous clinical trials with various types of cells have already been completed and many others are underway. However, and somewhat disappointingly, the efficacy of cell therapy toward inducing infarct repair has remained controversial. Given the differences in outcomes with regard to cardiac structure and function, it has been suggested that the results may be influenced by the choice of imaging techniques. In this chapter, we will provide an overview of cell therapy and discuss the advantages and disadvantages of imaging modalities that have been used in cell therapy clinical trials.

9.1 Evolution of Cardiac Cell Therapy

The concept that cells can be utilized to replace or repair injured myocardium emerged in the 1990s. At that time, it was believed that cardiomyocytes were terminally differentiated cells and incapable of further cell division. In one of the early preclinical reports, Marelli et al. sought to solve this problem by transplanting skeletal muscle satellite cells after myocardial injury induced by application of a cryoprobe in dogs [4]. The histological studies showed evidence of cellular retention at the sites of satellite cell injection in the scar area. In the subsequent years, several different types of cells were evaluated for their potential of myocardial integration and repair in different preclinical models of cardiac injury or uninjured hearts [5–10]. Around the same time, in a seminal paper in 1998, Anversa et al. contradicted the notion that ventricular myocytes are terminally differentiated by demonstrating that myocytes in adult mammalian hearts were capable of re-entering the cell cycle and undergoing cell division [11]. These observations led to great fervor in the scientific community, and fast-tracked cell-based therapy into clinical application [12–15].

The first use of cell therapy in humans dates back to a study reported in 2001, wherein Menasche et al. implanted autologous skeletal muscle myoblasts into the myocardial scar during coronary artery bypass surgery in a patient with ischemic heart failure [12]. At follow-up after 5 months, the investigators noticed evidence of contraction and viability in the grafted scar by echocardiography and PET. The field of cell therapy for ischemic heart disease has since greatly expanded to include numerous different types of cells in small clinical trials with each enrolling a small number of patients [13, 14, 16–19]. The designs of these trials have varied significantly from each other with regard to cell type, cell processing, number of

cells, route and timing of cell injection, recipient patient population, duration of follow-up, and mode of cardiac imaging. Although effective repair of infarcted and/or cardiomyopathic myocardium has been reported with several different cell types, BMCs have been utilized most widely for cardiac repair in patients with ischemic heart disease.

9.2 Bone Marrow Cell Therapy for Ischemic Heart Disease

The early success with BMCs for heart repair in animal models coupled with the ease of acquisition of these cells led to rapid translation of BMC therapy in humans. In early clinical studies, Hamano et al. injected autologous BMCs into the scar tissue of five patients with ischemic heart disease during coronary artery bypass graft surgery and followed them for a year [20]. They reported an improvement in myocardial perfusion by cardiac scintigraphy in three of the five BMC-treated patients. Strauer et al. injected autologous BMCs into the infarct-related artery after PCI in patients with acute MI and compared outcomes with a control group [13]. They reported reduced infarct size and improved myocardial contractility and perfusion in BMC-treated patients. Importantly, global LVEF and left ventricular end-diastolic volume (LVEDV) did not change significantly.

The BOne marrOw transfer to enhance ST-elevation infarct regeneration (BOOST) trial was the first randomized controlled trial (RCT) of BMC therapy for myocardial repair [21]. In this study, 60 patients were randomized to receive either BMC therapy or standard of care following percutaneous coronary intervention (PCI) for acute ST-segment elevation MI. After 6 months of follow-up, cardiac MRI showed increase in global LVEF by 0.7 percentage points in the control group and 6.7 percentage points in the cell therapy group, indicating that BMC therapy was associated with a significant improvement in cardiac function. However, after 18 months of follow-up, LVEF improved by 3.1 percentage points in the control group and 5.9 percentage points in the BMC group, but there was no significant difference between these groups [22]. These observations led to the notion that although BMC therapy significantly improves cardiac function during early follow-up, these advantages are eventually lost over longer term. The 18-month follow-up data from BOOST, however, did show an improvement in diastolic function in BMC-injected patients [23]; and the 5-year follow-up data showed significant improvement in cardiac function by MRI in BMC-treated patients with greater infarct transmurality [24].

With the rapid increase in the number of BMC trials for cardiac repair, the differences in outcomes with such therapy also became apparent. For example, the results from two RCTs, Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) and Autologous Stem-Cell Transplantation in Acute Myocardial Infarction (ASTAMI) trials, both of which used similar BMC populations in patients with acute MI, were remarkably different. REPAIR-AMI randomized 204 patients to receive either intracoronary injection of autologous BMCs or standard therapy at 3–7 days after PCI for acute MI. After 4

months of follow-up, the absolute improvement in LVEF in the BMC-treated group was significantly greater compared with the placebo group [15]. Importantly, they also reported a significant decrease in the combined endpoint of death, recurrent MI or necessity for any revascularization in the BMC group compared with controls at 1 year [15]. This indicated that administration of BMCs was safe and provided clinical benefits to patients. Contrary to these findings, the ASTAMI trial, which randomized 100 patients to receive either BMC injection or standard therapy after PCI for acute MI reported no significant benefit of BMC therapy [25]. In ASTAMI, change in cardiac function was assessed using SPECT, echocardiography and MRI at 6 months, which did not show any difference between cell-treated and control patients. After 3 years, only a small difference in exercise time was noted in the BMC group, however, without significant differences in LV functional change [26]. The differences in findings of the Repair-AMI and ASTAMI trials were attributed to the differences in cell processing and storage [27, 28].

Irrespective of the possible underlying reasons for differences among results from various trials, the outcomes of cardiac repair with BMCs have been clearly disparate. Although a large number of trials have shown multifaceted benefits with BMC injection in patients with ischemic heart disease, several trials have failed to show any significant improvement in cardiac parameters [16, 19, 29]. For example, the FOCUS-CCTRN trial, which included 92 patients, did not find any significant difference between the effects of BMC therapy and standard therapy on LVESV index (LVESVI), maximal oxygen consumption, reversible defect, percentage myocardial defect, total defect size, regional wall motion and clinical improvement at 6 months [30]. Similarly, the TIME trial failed to show any functional improvement as a result of BMC injection [31, 32]. Therefore, the efficacy of BMC therapy for cardiac repair continues to remain somewhat uncertain in view of differences in outcomes from small trials.

9.3 Meta-Analyses of Pooled Data from Clinical Trials of BMC Therapy

It is estimated that nearly 80 randomized controlled trials (RCTs) of BMC therapy for heart repair have already been completed and more than 30 are currently ongoing [33]. However, each of these clinical trials has been relatively unique with regard of cell type, cell number, cell processing technique, injection route, patient population, follow-up duration and other trial design considerations. The interplay of these variables makes it a challenging task to compare the results of any two apparently similar trials. In view of these facts, we performed the first comprehensive synthesis of clinical evidence in this nascent field in a systematic review and meta-analysis in 2007 [16]. In this meta-analysis, we analyzed cumulative data from 18 clinical trials enrolling 999 patients. The results showed modest yet significant improvements in LVEF, infarct size and LV end-systolic volume in BMC-treated

patients with acute MI or chronic IHD compared with controls, who received standard treatment. BMC therapy was not associated with any significant increase in adverse events. Similar beneficial effects of BMC therapy have since been reported in numerous meta-analyses examining various different aspects of this novel approach, thereby improving our understanding about the true potential of cell therapy for heart repair. Indeed, systematic reviews and meta-analyses can be very useful both for deciphering underlying trends that are otherwise inconspicuous, and for identifying potential biases. However, the findings of meta-analyses may also differ from each other, depending on the specific search criteria and inclusion and exclusion criteria for each specific study. For example, results from a recent individual patient data meta-analysis failed to identify any benefit associated with BMC therapy [34]. However, this particular meta-analysis included data from only 12 trials, whereas other large meta-analyses published to date have included data from more than 40 BMC trials [19, 29]. Despite these differences, large, well-conducted meta-analyses are highly valuable toward estimating the efficacy of treatment and generating hypotheses for future clinical trials.

Our group recently published the largest meta-analysis of RCTs evaluating the effects of BMC therapy in patients with IHD [19]. Data from 48 RCTs [15, 21, 22, 24–26, 30–32, 35–84] enrolling 2602 patients, comparing the effects of BMC injection in patients with acute MI or chronic ischemic heart disease (CIHD) were included in this meta-analysis. In these trials, patients received BMC therapy through intracoronary or intramyocardial routes and were followed up over time to assess safety, efficacy and clinical outcomes. Table 9.1 summarizes the characteristics of these included trials. Of note, the median sample size was 43 patients (range, 10–204 patients), median follow-up duration was 6 months (range, 3–60 months) and median number of BMCs injected was 125×10^6 (range, 2×10^6–60×10^9). In patients with acute MI, the timing of BMC injection varied greatly among studies (median 7 days after MI; range, 1–18 days). Meta-analysis of pooled data showed that compared with standard therapy, BMC transplantation improved LVEF by 2.92%, reduced infarct size by 2.25% and reduced LVESV by 6.37 ml. There was a trend toward reduction in LVEDV (−2.26 ml; 95% CI, −4.59 to 0.007; $P = 0.06$) in BMC-treated patients. Subgroup analysis revealed that improvement in LVEF, infarct size and LVESV persisted beyond 12 months during follow-up. Importantly, BMC transplantation resulted in significant improvements in LVEF, infarct size and LVESV in patients with both acute MI and CIHD. Patients benefitted from BMC therapy regardless of their baseline LVEF, although patients with a lower LVEF at baseline showed a greater improvement in LVESV. BMC transplantation in doses less than 50 million was shown to be ineffective. BMC transplantation 3–10 days after MI led to significant improvements in LVEF, LVESV and LVEDV but infarct size was reduced only when cells were transplanted within the first 48 h after MI.

Similar findings have been reported in meta-analyses that examined the outcomes of BMC therapy in different patient subpopulations. Delewi et al. performed a meta-analysis of 16 trials, enrolling 1641 patients, wherein patients received intracoronary BMC therapy following acute MI [85]. BMC therapy led to a 2.55% increase in LVEF compared with controls. LVESVI and LVEDV index (LVEDVI)

Table 9.1 Characteristics of RCTs included in meta-analysis [19]

Source	Sample size	Mean follow-up duration (months)	Cell type	No. of cells transplanted	Route of injection	Type of IHD	Imaging modalities
Ang et al. [35]	25	6	BMMNC	$85 \pm 56 \times 10^6$ (IM) $115 \pm 73 \times 10^6$ (IC)	IM or IC w/ CABG	CIHD	MRI
Assmus et al. [36]	46	3	BMMNC	$205 \pm 110 \times 10^6$	IC	CIHD	LVG
Bartunek et al. [37]	36	6	MSC	733×10^6 (605–1168)	Endomyo	CIHD	Echo
Cao et al. [38]	86	48	BMMNC	$5 \pm 1.2 \times 10^7$	IC	AMI	Echo (EF, Vol), SPECT (IS)
Chen et al. [39]	69	6	MSC	$48–60 \times 10^9$	IC	AMI	LVG (EF), PET (IS)
Colombo et al. [40]	10	12	CD133+ BMMNC	$5.9\ (4.9–13.5) \times 10^6$	IC	AMI	Echo (EF, Vol)
Gao et al. [41]	43	24	MSC	$3.08 \pm 0.52 \times 10^6$	IC	AMI	Echo
Ge et al. [42]	20	6	BMMNC	40×10^6	IC	AMI	Echo (EF), SPECT (IS)
Grajek et al. [43]	45	12	BMMNC	$2.34 \pm 1.2 \times 10^9$	IC	AMI	Echo
Hendrikx et al. [44]	20	4	BMMNC	$60.25 \pm 31.35 \times 10^6$	IM	CIHD	MRI
Hirsch et al. [45]	134	4	BMMNC	$296 \pm 164 \times 10^6$	IC	AMI	Echo, MRI
Huang et al. (abstract) [46]	40	6	BMMNC	NA	IC	AMI	MRI
Huikuri et al. [47]	80	6	BMMNC	$402 \pm 196 \times 10^6$	IC	AMI	Echo (EF), LVG (Vol)
Janssens et al. [48]	67	4	BMMNC	$172 \pm 72 \times 10^6$	IC	AMI	MRI
Jazi et al. [49]	32	6	BMMNC	$24.6 \pm 8.4 \times 10^8$	IC	AMI	Echo
Lipiec et al. [50]	36	6	BMMNC	$0.33 \pm 0.17 \times 10^6$ CD133+ $3.36 \pm 0.1.87 \times 10^6$ CD34+	IC	AMI	SPECT
Lu et al. [51]	50	12	BMMNC	$13.38 \pm 8.14 \times 10^7$	IC	CIHD	MRI
Lunde et al. [25, 52]; Beitnes et al. [26]	100	36	BMMNC	$87 \pm 47.7 \times 10^6$	IC	AMI	SPECT (EF, EDV, IS), Echo (ESV)

Maureira et al. [53]	14	6	BMMNC	1×10^7	Transmyo	CIHD	MRI
Meluzin et al. [54, 55]	66	12	BMMNC	High dose: 1×10^8 Low dose: 1×10^7	IC	AMI	SPECT
Meyer et al. [22, 24]; Wollert et al. [21]	60	18	BMMNC	$24.6 \pm 9.4 \times 10^8$	IC	AMI	MRI
Nogueira et al. [56]	20	6	BMMNC	1.0×10^8	IC	AMI	Echo
Penicka et al. [57]	27	4	BMMNC	26.4×10^8	IC	AMI	Echo (EF, Vol), SPECT (IS)
Perin et al. [58]	30	6	BMMNC	$484.1 \pm 313 \times 0\ 10^6$	Endomyo	CIHD	SPECT
Perin et al. [59]	20	6	BMMNC	$2.37 \pm 1.31 \times 10^6$	Endomyo	CIHD	Echo
Perin et al. [30]	82	6	BMMNC	$99.03 \pm 5.58 \times 10^6$	Endomyo	CIHD	Echo
Piepoli et al. [60, 61]	38	24	BMMNC	24.88×10^7	IC	AMI	Echo
Plewka et al. [62, 63]	56	24	BMMNC	$14.4 \pm 4.9 \times 10^7$	IC	AMI	Echo
Pokushalov et al. [64]	109	12	BMMNC	$41 \pm 16 \times 10^6$	IM	CIHD	Echo
Quyyumi et al. [65]	31	6	CD34+	$5–15 \times 10^6$	IC	AMI	MRI
van Ramshorst et al. [66]	49	3	BMMNC	100×10^6	IM	CIHD	MRI
Roncalli et al. [67]	101	3	BMMNC	$98.3 \pm 8.7 \times 10^6$	IC	AMI	MRI Echo
Ruan et al. [68]	20	6	BMC	NR	IC	AMI	Echo
Schachinger et al. [15, 69]; Assmus et al. [70]	204	4	BMMNC	$236 \pm 174 \times 10^6$	IC	AMI	LVG
Silva et al. [71]	30	6	BMMNC	1×10^8	IC	AMI	RNV
Srimahachota et al. [72]	23	6	BMMNC	$420 \pm 221 \times 10^6$	IC	AMI	MRI
Suarez de Lezo et al. [73]	20	3	BMMNC	$9 \pm 3 \times 10^8$	IC	AMI	LVG
Surder et al. [74]	133	4	BMMNC	$159.7 \pm 125.8 \times 10^6$	IC	AMI	MRI

(continued)

Table 9.1 (continued)

Source	Sample size	Mean follow-up duration (months)	Cell type	No. of cells transplanted	Route of injection	Type of IHD	Imaging modalities
Traverse et al. [31, 32]	95	12	BMMNC	150×10^6	IC	AMI	MRI
Traverse et al. [75]	87	6	BMMNC	$1.47 \pm 17 \times 10^8$	IC	AMI	MRI
Traverse et al. [76]	40	6	BMMNC	1×10^8	IC	AMI	MRI
Tse et al. [77]	28	6	BMMNC	$1.67 \pm 0.34 \times 10^7$ (low), $4.20 \pm 2.80 \times 10^7$ (High)	IM	CIHD	MRI
Turan et al. [78]	56	12	BMMNC	$99 \pm 25 \times 10^6$	IC	CIHD	LVG
Turan et al. [79]	62	12	BMMNC	$9.6 \pm 3.2 \times 10^7$	IC	AMI	LVG
Wohrle et al. [80, 81]	40	36	BMMNC	$381 \pm 130 \times 10^6$	IC	AMI	MRI
Yao et al. [82]	47	6	BMMNC	180×106	IC	CIHD	MRI
Yao et al. [83]	39	12	BMMNC	$1.9 \pm 1.2 \times 10^8$ (single transfusion), $2.0 \pm 1.4 \times 10^8$ (repeat transfusion)	IC	AMI	MRI
Zhao et al. [84]	36	6	BMMNC	$6.59 \pm 5.12 \times 10^8$	IM w/CABG	CIHD	Echo

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AMI acute myocardial infarction, *BMC* bone marrow cells, *BMMNC* bone marrow mononuclear cells, *CABG* coronary artery bypass graft, *CIHD* chronic ischemic heart disease, *Echo* echocardiography, *EF* ejection fraction, *Endomyo endomyocardial, IC* intracoronary, *IM* intramuscular, *IS* infarct size, *LVG* left ventriculography, *MI* myocardial infarction, *MRI* magnetic resonance imaging, *MSC* mesenchymal stem cells, *NA* not available, *NR* not reported, *PET* positron emission tomography, *Transmyo* transmyocardial, *RCT* randomized controlled trial, *RNV* radionuclide ventriculography, *SPECT* single-photon emission computed tomography

were significantly reduced by −2.60 ml/m^2 and −3.17 ml/m^2, respectively, following cell therapy. Improvement in LVEF and LVESVI was superior in patients under the age of 55 and in those with baseline LVEF <40%. Patients younger than 55 also showed a significant improvement in LVEDVI compared with older patients. Kandala et al. performed a meta-analysis of ten RCTs that included 519 patients, in the setting of chronic ischemic cardiomyopathy [86]. BMC therapy improved LVEF by 4.48%, reduced LVESV by 20.64 ml and LVEDV by 16.71 ml at 6 months compared with controls. However, the ACCRUE study, a meta-analysis based on individual patient data from 12 trials for cell therapy in patients with AMI, failed to identify any significant improvement in cardiac parameters with cell therapy [34].

9.4 Impact of Imaging Modalities on Outcomes of BMC Therapy

Although differences in other aspects of trial design may account for the observed variances in outcomes, the choice of imaging modalities has been implicated as one of the underlying reasons. A notion has been advanced that some of the benefits of BMC injection stem from the use of rather inaccurate imaging techniques that are inferior to MRI, the current gold standard. Irrespective of the superiority of one method over another, RCTs of BMC therapy have indeed utilized diverse imaging modalities to assess parameters of cardiac structure and function. Echocardiography, LVG, SPECT and MRI are the most commonly used techniques for the assessment of LVEF, infarct size, LVESV, and LVEDV, as shown in Table 9.1. These modalities vary considerably with regard to fundamental principles of imaging, and some are more appropriate in certain scenarios than others. For instance, functional and volumetric assessment by MRI is more reliable than by echocardiography [87]. Myocardial scar size measured by MRI closely agrees with PET data [88]. However, MRI also tends to overestimate infarct size soon after an MI due to the presence of tissue edema. Therefore, the assessment of infarct size by MRI was delayed until 2–3 weeks after the MI in select trials [25]. Table 9.2 summarizes the relative advantages and disadvantages of these cardiac imaging modalities. MRI is generally considered the gold standard for assessment of cardiac structure and function.

The various imaging methods utilized in BMC clinical trials are provided in Table 9.1. In these trials, echocardiography was almost always used due to its widespread availability and relative lack of contraindications. It is a fast and fairly accurate tool to assess cardiac structure and function. However, several trials chose more than one imaging modality so that LV structure and function could be assessed using the optimal techniques for respective parameters. For example, the FOCUS-CCTRN trial used echocardiography for LVEF and LVESVI assessment, but used SPECT to assess perfusion defect [30]. Confirmation of results with the use of another imaging technique reduces the possibility of assessments being affected by the type of imaging technique chosen. The ASTAMI trial used both echocardiography and MRI to assess LVEF, LVESV, and LVEDV [26], while the FINCELL trial

Table 9.2 Relative advantages and disadvantages of imaging modalities used in clinical trials of bone marrow cell therapy

Mode of imaging	Advantages	Disadvantages
Echocardiography	Easy availability, reproducibility, good correlation with MRI data	Potential inter- and intra-observer variability
Left ventriculography	Easy to perform during index procedure of cell transplantation	Invasive procedure, inconvenient for serial assessment of cardiac function. Potential inter- and intra-observer variability
SPECT	Superior pixel-based quantitative data for infarct size measurement	Radiation exposure. Gated data for volumetric assessment could be influenced by the underlying rhythm
MRI	Accurate volumetric assessment. Accurate infarct size assessment at follow up	Less widely available. Difficult to perform in patients with certain implanted cardiac devices or other implants

SPECT single-photon emission computed tomography, *MRI* magnetic resonance imaging

used both left ventricular angiography and echocardiography to estimate LVEF [47]. Interestingly, Traverse et al. reported that in their MRI laboratory, LVEF measured by MRI was 5–10% higher compared with LVEF measured by echocardiography, highlighting a quantitative difference in assessment of cardiac parameters that is based on the choice of imaging technique [76]. In this regard, several studies that utilized more than one imaging modality also reported data from these dissimilar techniques at various time-points. A comparative review of these numbers reveal that despite minor differences, results were consistent across multiple modes of imaging, thereby indicating no major or significant impact of the mode of imaging on study conclusions [26, 47, 67]. These observations are consistent with the results from studies that compared the accuracy of various cardiac imaging and reported a high degree of correlation among CT, echocardiography and MRI [89].

In order to determine whether the outcomes of BMC therapy were influenced by imaging techniques in clinical trials, we performed subgroup analysis based on data from LVG, echocardiography, SPECT and MRI (Table 9.3) [19]. Interestingly, our results showed that BMC therapy improved LVEF compared with controls when measured by echocardiography, LVG and MRI, but not SPECT. Similarly, LVESV was significantly reduced by BMC therapy when measured by echocardiography, LVG and MRI, but not SPECT. Infarct size was significantly decreased by SPECT analysis but not by MRI. LVEDV decreased significantly in BMC-treated patients when measured by echocardiography and SPECT, but not LVG and MRI. However, it is important to note that data from these diverse modalities are directionally concordant. Overall, these subgroup analyses indicate that BMC therapy improves cardiac function when measured by all imaging techniques, even though statistical significance was not reached for some parameters with certain modes of imaging perhaps due to smaller patient numbers.

Table 9.3 Unadjusted differences in mean change in outcome parameters in bone marrow cell-treated patients compared with controls based on the mode of imaging

Follow-up duration	BMC therapy (n)	Control (n)	Difference in mean [95% CI]	*P* Value for Z	*P* Value for subgroup differences
LVEF					
Echo	642	533	2.69 [1.27, 4.12]	0.0002	0.05
SPECT	150	96	0.93 [−0.83, 2.68]	0.30	
MRI	642	533	1.60 [0.30, 2.90]	0.02	
LVG	372	305	5.26 [2.47, 8.05]	0.0002	
Infarct size					
SPECT	155	135	−2.41 [−2.78, −2.03]	<0.00001	0.19
MRI	416	306	−1.18 [−2.97, 0.61]	0.20	
LVESV					
Echo	249	195	−11.76 [−19.09, −4.43]	0.002	0.02
SPECT	116	80	−4.57 [−11.13, 1.99]	0.17	
MRI	341	252	−2.59 [−3.90, −1.27]	0.0001	
LVG	211	176	−11.12 [−19.27, −2.97]	0.008	
LVEDV					
Echo	283	235	−2.41 [−2.79, −2.03]	0.00001	0.42
SPECT	116	80	−9.56 [−18.39, −0.72]	0.03	
MRI	391	302	−1.48 [−6.21, 3.25]	0.54	
LVG	211	176	−0.31 [−10.47, 9.86]	0.95	

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Echo echocardiography, *LVEDV* left ventricular end-diastolic volume, *LVEF* left ventricular ejection fraction, *LVESV* left ventricular end-systolic volume, *LVG* left ventriculography, *MRI* magnetic resonance imaging, *SPECT* single-photon emission computed tomography, *CI* confidence interval, *n* number of patients in each group

Although recent RCTs of cell therapy have largely utilized MRI, the above evidence supports the applicability of all imaging techniques in a comparable fashion in cell therapy trials. However, with regard to selection of imaging method(s), additional important factors need to be considered. Many trials cannot afford to use MRI due to its relative lack of availability and higher cost. Furthermore, many patients who enroll in these clinical trials have poor cardiac function resulting from complex cardiac pathologies and may be clinically unstable. These patients may also have implantable

electronic cardiac devices. These conditions pose contraindications for MRI, and trial investigators may have to either exclude these patients from the analysis or use other imaging techniques to incorporate these patients in their trials. Together, these factors weave a complex scenario wherein the ultimate choice of imaging technique for cell therapy trials may be made for reasons other than reliability and reproducibility of data, i.e. cost, availability, feasibility, and patient characteristics.

A review of the FOCUS-CCTRN trial highlights the practical implications of the above points. In this study, 92 patients with ischemic cardiomyopathy were randomized in a 2:1 ratio with 62 patients scheduled to receive BMC therapy and 31 standard therapy [30]. Of these patients, 54 and 28 patients, respectively, underwent echocardiography evaluations at baseline and at 6 months. SPECT for perfusion defects was performed on 52 and 25 patients respectively. Surprisingly, only 17 of the 92 initially randomized patients were without contraindications for MRI. The investigators were unable to obtain meaningful data from MRI analysis due to the large number of patients, who were excluded due to ineligibility for MRI evaluation.

9.5 Assessment of Clinical Outcomes

Although accurate measurement of cardiac structure and function is important toward determining the efficacy of cell therapy, assessment of impact of such therapy on clinical events is perhaps more important. Indeed, data generated over 15 years of clinical cardiac cell therapy have consistently proven that bone marrow cells are safe for use in humans. As the injected cells are mostly autologous, they do not generate an immune response. Mere transference of these cells from the bone marrow to the heart has not been associated with any major adverse effects. Besides safety, clinical outcomes also determine the long-term efficacy of this emerging therapy. However, the incidence of adverse clinical events is often low, and given the relatively small number of patients in individual trials, analysis of clinical outcomes from pooled data in meta-analysis has been particularly helpful.

In the most recent meta-analysis, our data indicated that BMC therapy was associated with significant reduction in all-cause mortality, recurrent MI, ventricular tachycardia/ventricular fibrillation and CVA/transient ischemic attack compared with standard therapy [19]. There were trends toward reduction in cardiac death, heart failure and stent thrombosis, although these differences did not reach significance (Table 9.4). The lack of increase in stent thrombosis and in-stent restenosis in BMC-treated patients was particularly encouraging, since BMC injection in the infarct-related artery has been shown to increase atheroma burden [90]. On the other hand, the recently reported ACCRUE study failed to show any improvement in clinical outcomes following cell transplantation [34]. In view of these differences, the results of the currently ongoing phase III Effect of Intracoronary Reinfusion of Bone Marrow-derived Mononuclear cells on All Cause Mortality in Acute Myocardial Infarction (BAMI) trial are likely to provide a definitive answer regarding the efficacy of BMC therapy on patient survival.

Table 9.4 Clinical outcomes in bone marrow cell-treated patients compared with patients receiving standard therapy

Outcome	BMC therapy (n)	Control (n)	Peto OR [95% CI]	*P* Value
All-cause mortality	1397	980	0.55 [0.34–0.89]	0.01
Cardiac deaths	970	666	0.52 [0.24–1.13]	0.10
Recurrent MI	1159	799	0.50 [0.27–0.92]	0.03
Heart failure	1027	761	0.62 [0.37–1.05]	0.08
Stent thrombosis	599	458	0.48 [0.21–1.09]	0.08
In-stent restenosis	432	332	0.92 [0.55–1.54]	0.75
TVR	866	606	0.84 [0.59–1.21]	0.36
CVA	640	462	0.25 [0.08–0.81]	0.02
VT/VF	481	419	0.45 [0.22–0.93]	0.03

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BMC bone marrow cell, *CVA* cerebrovascular accident, *MI* myocardial infarction, *OR* odds ratio, *TVR* target vessel revascularization, *VF* ventricular fibrillation, *VT* ventricular tachycardia, *CI* confidence interval, *n* number of patients in each group

9.6 Conclusions

Cell therapy represents a highly promising approach for effective cardiac repair in patients with ischemic heart disease and cardiomyopathy. Although results from smaller clinical trials of BMC therapy have been disparate, evidence from a large number of meta-analyses suggests that BMC-treated IHD patients experience modest yet significant improvements in cardiac structure and function compared with patients receiving standard treatments. Although diverse imaging modalities have been used to measure specific LV parameters, meta-analysis of pooled data reveals directionally concordant results with different techniques. However, the selection of a specific imaging technique for a particular clinical trial depends on other factors besides accuracy. These include the availability of imaging equipment, cost, and patient characteristics. Moreover, the efficacy of BMC therapy for cardiac repair is also supported by imaging-independent parameters, such as improved clinical outcomes, including survival. Future large RCTs will likely provide definitive answers in this controversial yet critically important field.

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Chapter 10
Uterine Stem Cells and Their Future Therapeutic Potential in Regenerative Medicine

K.G. Aghila Rani and Taruna Madan

10.1 Introduction

Recent years have witnessed tremendous progress in using 'Adult Stem Cells' (ASCs) in regenerative medicine. ASCs are considered to be safer for therapeutic use and several are already in clinical trials. The presence of ASCs has been reported in a variety of human tissues like blood, heart, liver, intestine, muscles, skin, nervous system, dental pulp, adipose tissue, synovial membrane, umbilical cord blood, amniotic fluid and recently in the endometrium (lining of the uterus) [1]. Major advances have been made since then to identify the cells with progenitor/stem cell like activity in human and mouse endometrium and efforts are still ongoing to fully characterise these cells for their utility in therapeutic applications. Characteristic properties such as accessibility, ease of harvesting and diverse differentiation potential brands endometrial stem cells as a valuable source of autologous stem cells for regenerative medicine. On the other hand, the role of these putative stem cell populations has been associated with pathogenesis of certain gynaecological disorders involving abnormal cell proliferation such as endometriosis, endometrial hyperplasia, endometrial cancer and adenomyosis [2, 3]. This review focuses on identification of different populations of uterine/endometrial stem cells (EnSCs) and efforts evaluating their therapeutic utility for clinical applications.

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10.2 Origin of Endometrial Stem Cells (EnSCs)

Human endometrium is a dynamic organ, originated from the Mullerian ducts during embryonic life while the myometrium with a non-Müllerian origin, is developed during fetal life [4]. The endometrium is structurally and functionally divided into two major compartments: [5] the functionalis, transient layer containing glands extending from the surface epithelium and supportive stroma, and [6] the basalis, comprising the basal region of the glands, stroma, supporting vasculature, and lymphoid aggregates. Endometrium undergoes rapid cycles of regeneration that includes cellular proliferation, differentiation, and shedding of the functionalis layer during each round of menstrual cycle [7]. Although the underlying mechanisms are poorly understood, this cyclic renewal is likely to be contributed by a residing uterine stem cell population [8, 9]. It is believed that the stem cells residing in the basal endometrial layer [1] migrate to the functional layer and actively participate in the regeneration and remodelling of the endometrium (Fig. 10.1).

Though there are evidences for the presence of regenerating endometrial stem cell populations, their exact origin remains poorly understood [7]. The origin of endometrial stem cells is attributed to a number of possibilities such as [5] they are the fetal epithelial and mesenchymal stem cells (MSCs) which remain in the adult endometrium and continues to replicate in adulthood [6], the circulating bone marrow stem cells that seeds the endometrium either periodically or in response to injury, or [10] a combination of both [2, 9, 11, 12]. Several research groups have attempted at harvesting these regenerating, clonogenic stem cell populations from endometrial tissues.

EnSCs can be distinctly classified as epithelial progenitor cells, MSCs, endothelial progenitor cells and endometrial side population cells [1].

10.2.1 Epithelial Progenitor Cells

Epithelial progenitor cells reside in the basalis layer of the endometrium and are not shed during the menstrual cycle [13]. Menstrual blood contains only stromal cell types and lacks epithelial cells [13]. The findings corroborate with earlier reports that clonogenic cells are present both in the active and inactive endometrium, as inactive endometrium is predominantly basalis and lacks functionalis layer [14].

10.2.2 Mesenchymal Stem Cells

The presence of clonogenic MSC populations has been detected in the endometrium of peri-menopausal women, post-menopausal women, and women on oral contraceptives [14]. Techniques involved in isolation of MSCs from endometrial biopsy

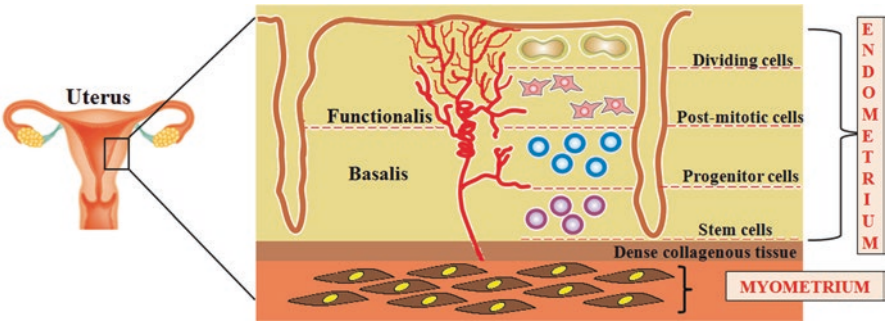


Fig. 10.1 Localisation of endometrial stem cells in human endometrium

specimens have been well described [15–17]. Briefly, endometrial tissue biopsies were subjected to enzymatic and mechanical dissociations and diluted into single-cell suspensions of stromal and epithelial cells. The dissociated cells were further filtered through sterile 40-μm cell strainers to separate stromal cells that consist of mainly the flow through and the undigested epithelial glandular clumps were retained in the strainer. Regenerating stem cell clones were developed from both stromal and epithelial cells [15]. Endometrial MSC clones were generated by either direct dilution of pure stromal cell isolates using serial dilution techniques [15] or by single cell cloning of culture grown stromal cells following five repeated passages ([17]; Fig. 10.2). Endometrial MSCs can be grown extensively both in vivo and in vitro and maintained in culture for up to 40 passages. They exhibit a diverse differentiation potential on specific induction conditions such as chondrogenic, osteogenic, adipogenic, angiogenic and myogenic lineages [1, 17].

10.2.3 Endothelial Progenitor Cells

The progenitor cells in the endometrium are reported to have high proliferative potential and can generate upto 6×10^{11} cells from a single cell [15]. These cells were able to differentiate into large cytokeratin-expressing structures when cultured in matrigel and were used as a substitute for mouse embryonic fibroblast feeder layers in embryonic stem cell cultures [15].

10.2.4 Endometrial Side Population (SP) Cells

A number of studies suggest that endometrial stem cells possess side population (SP) phenotype that is characterized by their ability to exclude the DNA-binding dye Hoechst 33343 due to presence of ATP-binding cassette transporter proteins [18]. Endometrial SP cells were identified both in the epithelial and stromal

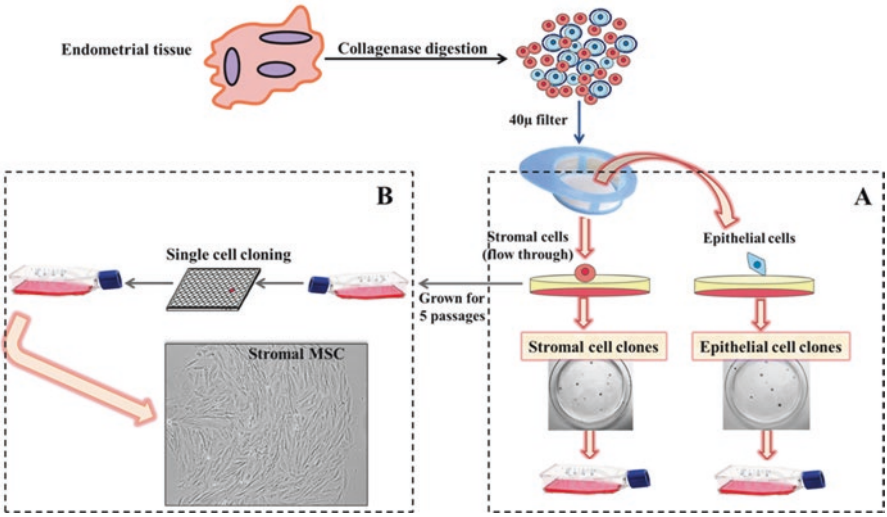


Fig. 10.2 Isolation and culture of endometrial stem cells. The image represents stepwise isolation procedures of endometrial stem cells by two different methods; (**a**) direct dilution and selection of colony forming units and (**b**) single cell cloning of culture grown (passage 5) stromal cells

compartments of the endometrium [19, 20]. They were able to differentiate in vitro into adipogenic and osteogenic lineages and developed human endometrium on subcutaneous injection in NOD-SCID mice [11, 12, 19].

10.2.5 Endometrial Regenerative Cells (ERC)

Recent studies identified the presence of stem -cell like population in menstrual blood [21, 22]. These cells termed as Endometrial Regenerative Cells (ERC), are plastic adherent and were able to maintain in tissue culture for >68 population doublings. They expressed known stem cell markers such as Oct-4, SSEA-4, c-kit, CD9, CD29, CD41a, CD44, CD59, CD73, CD90 and CD105. Proliferative potential of these cells was significantly higher when compared to umbilical cord derived MSCs with a doubling time of 19.4 h. ERCs exhibited a differentiation potential to nine varied lineages such as cardiomyocytic, respiratory epithelial, neurocytic, myocytic, endothelial, pancreatic, hepatic, adipocytic, and osteogenic [23]. Studies suggest that ERCs are easily expandable in culture and thus could serve as plausible tools in future regenerative medicine.

10.3 Identification of EnSCs in Murine Models

The presence of regenerating EnSC populations in murine models was detected using label-retaining cell (LRC) approach, wherein animals were injected with the thymidine analogue, bromodeoxyuridine (BrdU), which incorporates into genomic DNA during the replication phase of mitosis. The tissue of interest was examined for cells which retain this label after a prolonged chase period. Though there are evidences supporting the existence of a small population of uterine stem cells in mouse using the LRC approach, their exact location is unclear [7]. Chan and Garget reported that around 3% of epithelial cells and 6% of stromal cells adjacent to the luminal epithelium at the endometrial myometrial junction are LRCs [24]. Also, these epithelial and stromal LRCs differentially express estrogen receptor-1 (Esr1 or ERα) [24]. Contrary to this report, Cervelló et al. reported that LRCs are present only in the stromal compartment and not in the epithelial compartment [25]. Yet another study reported lack of LRCs in the stromal cell compartment [26] but identified epithelial LRCs in the glandular epithelium. The discrepancies in existing data warrant a detailed study on understanding the source of regenerative endometrium in murine models.

10.4 Markers Identifying Human EnSCs

Human EnSCs show positivity for expression of bone marrow stem cell markers like CD9, CD13, CD14, CD29, CD31, CD44, CD73, CD90, CD105, CD117, CD133, CD146 [27], but negative for STRO-1, CD31 (endothelial) and CD34 (haematopoietic stem cell and endothelial) markers [28]. Majority of the studies till date follow the above panel for characterising the isolated EnSC populations. Separation and purification of endometrial MSC-like cells (eMSCs) is reported to be efficient on basis of their co-expression of two perivascular markers, cluster of differentiation 140b (CD140b; platelet-derived growth factor receptor b (PDGFRb]) and CD146 [29]. Other markers such as Musashi-1(neural stem cell marker), NAC1 (embryonic stem cell marker [30], MSI1 and NOTCH1, which maintain stem cells in an undifferentiated state, are also reported to be expressed by EnSCs [1]. Tissue non-specific alkaline phosphatase, leucine rich repeat containing G protein-coupled receptor-5 (Lgr-5) [31, 32] and W5C5 [33] are yet another set of markers that localize to a perivascular location in human endometrium and may be useful for the prospective isolation of EnSCs. Nevertheless, current research lacks evidence for a single specific marker for identifying endometrial stem/progenitor cells that distinguish them from their mature progeny. Hence, studies exploring stem cell isolation from endometrial tissues rely on using a cocktail of markers and functional properties of stem cells such as clonogenecity, proliferative potential and differentiation into one or more lineages [7].

10.5 Multi-dynamic Properties of EnSCs

10.5.1 Clonogenecity

The clonogenic potential of EnSCs was first reported by Chan et al. [1]. Epithelial and stromal cells were separated into single cell suspensions and cultured at a cloning density of 300–500 cells/cm^2 in different culture conditions such as in the presence of serum containing medium or serum-free medium supplemented with different growth factors. Cloning efficiencies of epithelial and stromal cells were reported to be 0.22% and 1.25% respectively. Thirty-seven percentage of epithelial colonies were small, with large, loosely arranged cells whereas 1 in 60 of stromal colonies were large colonies, comprising small, densely packed cells. Cloning of MSCs obtained from endometrial stromal cells described by other groups were different from the technique followed by Chan et al. wherein pure cultures of endometrial stromal cells were subjected to serial dilution and single cell cloning [1]. In other reports, passage 5 stromal cells were serially diluted in 96 well culture plates in order to obtain a cell count of one cell/well. Dividing clonal populations obtained from single cell cultures of stromal cells were further cultured as regenerating MSCs [16, 17]. There was no variation in clonogenecity of EnSCs isolated from epithelial and stromal cells along the different stages of the menstrual cycle, i.e., from proliferative to secretory stage or between active, cycling and inactive endometrium [1].

10.5.2 Immunogenicity

Immunosuppression is one of the hallmark features of MSCs [34, 35] and the immuno-modulatory properties of MSCs isolated from different tissues were reported in several experimental settings [6, 10, 36, 37]. This cardinal ability of MSCs helps them in curbing many immune disorders [34, 37, 38]. Several studies have shown that MSCs in culture can mediate suppression of T-cell proliferation [34]. Immunosuppressive property of MSCs could be validated in vitro using PBMC proliferation assays [5]. Briefly, mitogen activated PBMCs are co-cultured with MSCs at a ratio of 10:1 for 48 h in multi-well culture plates. MTT assay is performed following incubation and percent change in cell proliferation is validated. Percent change in suppression of PBMC proliferation is calculated using the formula: % change = ({(mean OD of triplicate wells of PBMC + PHA) − (mean OD of triplicate wells of PBMC + MSC + PHA)}/(mean OD of triplicate wells of PBMC + PHA)) × 100. Immunosuppressive property of endometrial stromal cell derived MSCs has been validated in a recent study from our laboratory [17]. In vitro co-culture experiments involving healthy human endometrial MSCs (eutopic MSCs) resulted in approximately 50% reduction in proliferation of mitogen activated PBMCs [17].

10.5.3 Differentiation

Several studies have reported the potency of EnSCs, right from the endometrium of the fetus to the postmenopausal period, to differentiate to adipogenic, osteogenic and chondrocyte lineages, another hallmark feature shown by MSC populations [17, 39, 40]. These differentiated cells were identified by positive staining with Oil Red O (for adipogenesis), alizarin red (for osteogenesis), von Kossa (for calcified extracellular matrix), and Alcian blue (for sulfated proteoglycans) [23]. The differentiation and proliferation potential of human endometrial cells was attributed to the modulation of p38 and c-jun pathways [41].

10.6 EnSCs: Association with Disease Pathogenesis

Several gynaecological conditions are associated with abnormal endometrial proliferation. It is possible that endometrial stem/progenitor cells may play a role in the pathophysiology of such diseases such as endometriosis, endometrial hyperplasia, endometrial cancer and adenomyosis [2]. Alteration in the number, function, regulation and location of EnSCs could possibly be responsible for these endometrial diseases. Recent studies highlight the possible role of endometrial stem/progenitor cells in the origin of ectopic endometrial tissue in endometriosis, a major gynecologic concern affecting women in reproductive age [9]. Leyendecker et al. [42] showed that relatively more basalis layer of the endometrium was shed in the menstrual flow of women with endometriosis compared with that of normal controls. With the view that the endometrium basalis contains endometrial/stem progenitor cells and that women with endometriosis have larger volumes of retrograde menstrual flow, the data by Leyendecker et al. suggested that endometriotic implants result from the retrograde menstruation of endometrial stem/progenitor cells.

Studies by Kao et al. provided further evidence for the concept that endometriosis is the abnormal growth of endometrial cells sustained by stem cells with high invasive ability [16]. Kao et al. identified two different endometrial MSC populations in women with endometriosis. (1) The eutopic or healthy endometrial MSCs and (2) the ectopic or endometriotic MSCs [16]. Although both eutopic and ectopic MSCs showed similar mesenchymal cell phenotypes, ectopic endometrial MSCs showed distinctly greater ability of cell migration and invasion and when grown on scaffold and transplanted in immune-deficient mice, formed many new blood vessels and invaded surrounding tissues than eutopic MSCs.

In another study, Forte et al. reported the expression pattern of a panel of 13 stemness related genes in human endometrial and endometriotic tissues [43]. The study concluded that expression of *UTF1*, *TCL1* and *ZFP42* showed a trend for higher frequency of expression in endometriosis than in endometrium, while *GDF3* showed a higher frequency of expression in endometrial samples. The study further suggested a possible role of SALL4-positive cells in the pathogenesis of endometriosis

[43]. The hypothesis that stem/progenitor cells contribute to pathogenesis of endometriosis, may account for all existing theories explaining the origin of endometriotic implants. The retrograde menstruation theory can be supported by the detection of stem cells in the basalis layer of the endometrium that are shed through the fallopian tube to establish endometriotic implants. The coelomic metaplasia theory can be supported by the findings locating stem cells derived from the bone marrow in the endometrium. Embryonic rest theory can be supported by the presence of stem/progenitor cells that persist in the remnants of the mullerian system that form endometriotic implants. Bone marrow derived stem/progenitor cells are likely to travel to distant ectopic sites via the lymphovascular spaces supports the theory of lymphatic spread.

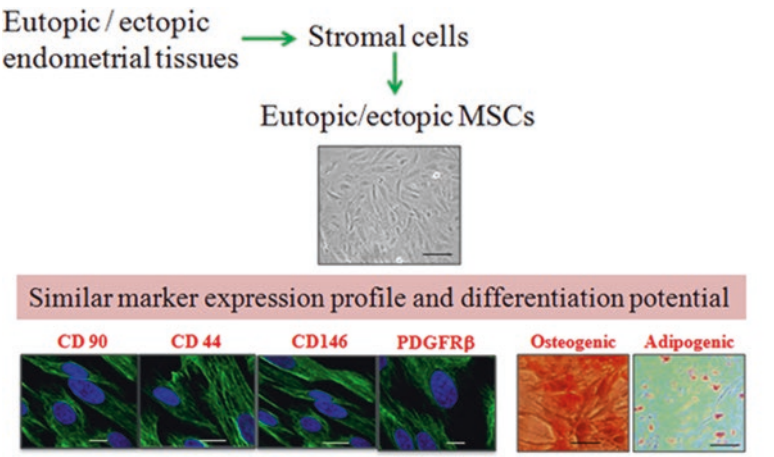
Recently, we reported several immuno-phenotypic differences between healthy endometrial (eutopic) and endometriotic (ectopic) MSCs [17]. The study addressed differential gene expression for an array of pattern recognition receptors (PRRs) and pro-inflammatory cytokines along with markers of migration and angiogenesis among eutopic and ectopic MSCs. Our findings suggest that, though these two cell types exhibit similar characteristic markers and differntiation potentials, ectopic MSCs possess an increased level of TLRs, collectins, pro-inflammatory cytokines and migration and angiogenesis markers. They exhibit a distinct immune-phenotype compared to eutopic MSCs. This differential immunophenotype plausibly contribute to the reduced immunosuppressive property of ectopic MSCs and thereby pathogenesis of endometriosis (Fig. 10.3). However, further in depth molecular studies are warranted to identify the differentially expressed factors of eutopic and ectopic MSCs to conclude the exact role of stem cells contributing the pathogenesis of endometriosis.

10.7 Therapeutic Utility of Uterine Stem Cells

The ease in availability of uterine stem cells marks its potential to be used in regenerative medicine and autologous stem cell therapies. A number of studies report the therapeutic utility of EnSCs in several in vitro and in vivo studies including a few clinical trials that have opened a new window in the history of regenerative medicine (Tables 10.1 and 10.2).

10.7.1 Eutopic MSCs for Targeted Delivery of Anti-angiogenic Agents in Endometriosis

Recently, we explored the therapeutic utility of eutopic MSCs for devising a targeted therapy for endometriosis (unpublished data). In the study, we demonstrated that eutopic MSCs could be genetically manipulated for expressing an anti-angiogenic factor, soluble FLT-1. Genetically manipulated eutopic MSCs expressed and secreted sFlt-1, and their therapeutic anti-angiogenic ability was validated in a



Dissimilar immune-phenotype	
Eutopic MSCs	Ectopic MSCs
PRR and Collectins *	PRR and Collectins ***
Pro-inflammatory cytokines *	Pro-inflammatory cytokines ***
MMPs *	MMPs ***
Angiogenesis ↑	Angiogenesis ↑↑↑
Immunosuppression ↑↑	Immunosuppression ↑

Fig. 10.3 A comprehensive overview of the phenotypic, differentiation and immuno-phenotypic characteristics of eutopic and ectopic MSCs. EnSCs showed positivity for expression of markers such as CD 90, CD 44, CD 146 and PDGFRβ. Under appropriate differentiation conditions, EnSCs differentiated to respective lineages as identified by indicated staining methods. Alizarin red staining for osteogenic and Oil-red staining for adipogenic lineages (*red colour* indicates lipid vacuoles stained by Oil-Red)

SCID mouse endometriosis (EM) model. Intravenous administration of therapeutic MSCs in EM models, resulted in reduced lesion growth and angiogenesis and exhibited impaired expression for VEGF and MMPs.

10.7.2 Myogenic Differentiation Potential of EnSCs

Menstrual blood-derived EnSCs hold high replicative ability and growth rate [61]. In vitro studies showed the myogenic differentiation potential of endometrial tissue derived cells obtained from human menstrual blood [44]. The cells expressed markers of skeletal myogenic differentiation such as MyoD, desmin, and myogenin upon

Table 10.1 In vitro studies on differentiation of human EnSCs

In vitro studies			
Lineage	Agent	Markers identified	References
Myogenic	5-Azacytidine	MyoD, desmin, and myogenin	[44]
Cardiac	Co-culture with fetal cardiomyocytes	Cardiac troponin and alpha-actinin	[45]
Neuronal	Biocompatible/biodegradable nanofibrous scaffolds seeded with EnSCs	Beta-tubulin III, islet-1, neurofilament-H, HB9, Pax6, and choactase	[46]; [47]
	Fibrin gels + EnSC derived neuron-like cells	B-Tubulin III and NF-L choline acetyltransferase, microtubule associated protein 2, neurofilament L	[48]
	EnSCs + growth factors - NGF and bFGF		[49]
	Co-culture with OGD exposed primary neurons	VEGF, BDNF, and NT-3	[50]
Pancreatic	Extracellular matrix supplemented with induction factors	PAX4, PDX1, GLUT2 and insulin	[51]
	Serum-free modified pancreatic selection medium	NKx2.2, Glut2, insulin, glucagon, somatostatin and c-peptide	[52]

induction with 5-azacytidine [44]. In vivo regenerative potential of these cells was studied in an experimental model of Duchenne muscular dystrophy (DMD) disease in immunodeficient (mdx) mice. DMD is a devastating genetic disorder characterized by progressive muscle degeneration and weakness owing to lack of dystrophin expression at the sarcolemma of muscle fibres [62]. When injected into the right thigh muscle of experimental DMD models, EnSCs contributed to recovery of dystrophin expression and subsequent muscle repair. The authors attributed the regeneration of muscle fibres to two different mechanisms: (1) myogenic differentiation of implanted or transplanted cells and/or (2) cell fusion of implanted or transplanted cells with the host muscle cells. Promising results provided by the study thus suggested the contribution of menstrual blood derived EnSCs towards cell-based therapies for muscle injury or chronic muscular disease.

10.7.3 Cardiac Regeneration Potential of EnSCs

EnSCs appear to be a potential novel, easily accessible source for cardiac regeneration therapy ([59]). MSCs derived from the endometrial glands when engrafted into recipient hearts of nude rats transdifferentiated into cardiac cells in vivo [45]. Upon induction in specific culture conditions, these cells began beating spontaneously, exhibited cardiomyocyte-specific action potential and also expressed cardiac

Table 10.2 In vivo studies and clinical trials employing human EnSCs

	In vivo studies				
	Disease model	Mode of administration	Animal model	Outcome	References
Cardiac regeneration	DMD	Intramuscular	DMD mouse	Recovery of dystrophin expression and subsequent muscle repair	[44]
	MI	Intra-myocardial	Nude rat	Transdifferentiation into cardiac cells and restoration of cardiac function	[45]
	MI	Intra-myocardial	Mouse	Improvement of myocardial function	[53]
Neural regeneration	MI	Intravenous	Rat	Homing to the injured myocardium, tissue repair and prevention of cardiac dysfunction	[54]
	Stroke	Intracerebral, intravenous	Rat	Significant reduction in behavioural and histological abnormalities of stroke	[50]
Pancreatic regeneration	Multiple sclerosis	Intraperitoneal	Mouse	Reduced EAE score; upregulated levels of anti-inflammatory cytokines	[55]
	Parkinson's disease	Intrastriatal	Primate	Development of neuron-like morphology; expression of tyrosine hydroxylase	[56]
	Diabetes	Renal subcapsular injection	SCID mouse	Blood glucose levels were stabilized within 5 weeks	[51]
	Diabetes	Renal subcapsular injection	SCID mouse	Blood insulin levels restored with prolonged survival of graft cells	[52]
Tissue engineering					
Scaffold	**Animal model**		**Outcome**		**References**
Gelatin-coated polyamide knit	Rat model of wound healing		↑ Neovascularization, tissue integration, collagen fibres, distensibility of the mesh; ↓chronic inflammation, fibrosis		[1]
Biomimetic gelatin/ apatite scaffolds	Rat model of bone regeneration		↑ Bone formation and maturation		[57]

(continued)

Table 10.2 (continued)

	In vivo studies				
	Disease model	Mode of administration	Animal model	Outcome	References
Clinical trials					
Disease condition	**Cell type used**	**No. of patients enrolled**	**Route of administration**	**No. of cells administered**	**Outcome**	**References**
Multiple sclerosis	ERCs	4	Intravenous and intrathecal	$16–30 \times 10^6$	No immunological reactions or adverse side effect on 1 year follow up	[58]
DMD	ERCs	1	Intramuscular	116×10^6	↑ Muscle strength and ↓ respiratory infection on 3 year follow-up	[59]
Ischemic cardiomyopathy	Allogeneic CD34 cells + ERCs	1	Intravenous	15×10^6	No mass formation, inflammation. EF increased from 30% to 40%, ↓ basic natriuretic peptide values	[60]

specific markers such as cardiac troponin and alpha-actinin. Transplanted cells were also able to restore cardiac function and significantly reduced the infarct area. Interestingly, the cardiomyogenic differentiation potential of EnSCs was observed to be higher when compared to bone marrow derived stem cells (BMSCs) [45]. This would possibly be due to the high angiogenic potential of EnSCs.

Homing of uterine stem cells to the infarcted heart was shown in murine [53] and rat models of myocardial infarction (MI) [54]. Ludke et al. demonstrated homing of uterine SCs to the myocardium in mouse models of myocardial infarction. The study showed that allogenic transplantation of uterine stem cells by intramyocardial injection resulted in significant improvement of myocardial function with comparable regenerative efficiency to syngenic bone marrow cell transplantation. Further, there was limited recruitment of CD4 and CD8 cells to the myocardial site suggesting the immunosuppressive properties of uterine SCs. Additionally, these cells exhibited significantly higher angiogenic potential that favoured the neovascularisation process, effectively aiding to healing of the infarct tissue.

In a study by Xaymardan et al., creation of MI in hysterectomised rats by coronary ligation post 7 days of heterotopic transplantation of uterus from GFP rats, resulted in detection of GFP (+) cells in the recipient hearts [54]. These cells were found to be present 7 days post MI and persisted for 6 months. Intravenous delivery of GFP + uterine cells immediately after MI also resulted in successful homing of injected cells to the injured myocardium. These cells were able to home to the injured myocardium, enhance tissue repair, and prevent cardiac dysfunction [54].

10.7.4 Neural Regeneration

Cell based restorative treatments for neural regeneration has gained importance over recent years and increasing data has shown promise and strong evidences for use of such means in managing CNS diseases [50, 55]. Several studies demonstrated that EnSCs have potential for neuronal differentiation. Differentiation of EnSCs to neurons is largely aided by the use of biocompatible and biodegradable nanofibrous scaffolds [46, 47], fibrin gels [48] and external growth factors such as NGF (nerve growth factor) and bFGF (basic fibroblast growth factor) [49]. EnSC differentiated neuronal cells expressed markers for neuronal regeneration such as beta-tubulin III, islet-1, neurofilament-H (NF-H), HB9, Pax6, and choactase. Comparative studies showed that expression of these neuronal markers were higher in EnSC differentiated cells when compared to bone marrow MSC differentiated neuronal cells [47]. EnSCs have shown promise in treating a variety of neurodegenerative conditions such as stroke, multiple sclerosis and parkinson's disease.

10.7.4.1 Stroke

Ischemic stroke is a leading cause of death and cell based therapy offers a new avenue in its treatment and care [50]. The condition could be mimicked in vitro using oxygen glucose deprivation (OGD) model system. Borlongan et al. reported that OGD -exposed primary rat neurons when co-cultured with menstrual blood-derived stem cells or exposed to the conditioned media of menstrual blood EnSCs, achieved significant protection against ischemic cell death. Elevated levels of certain trophic factors such as VEGF, brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) was also observed in the media of OGD-exposed menstrual blood-derived EnSCs. Based on these findings, the authors conducted an in vivo trial by transplanting EnSCs in a rat model of ischemic stroke. Intracerebral (IC) and intravenous (IV) injections of EnSCs significantly reduced behavioural and histological abnormalities of stroke. In vitro and in vivo assessments of the study revealed the efficacy and safety of transplanting menstrual blood-derived stem cells in stroke and probably their potential in treating other CNS disorders.

10.7.4.2 Multiple Sclerosis

The immunosuppressive property of EnSCs and their potential contribution in reducing neuroinflammation has been demonstrated in a murine model of multiple sclerosis (MS) [55], a neurodegenerative condition affecting the central nervous system. Experimental autoimmune encephalomyelitis (EAE) is the most commonly used experimental model for studying MS. The disease involves interaction between a variety of immunopathological and neuropathological mechanisms aiding to certain key pathological features of MS such as inflammation, demyelination, axonal loss and gliosis [63]. Intraperitoneal delivery of EnSCs exerted a potential anti-inflammatory effect in EAE models [55]. Lowered levels in number of infiltrating mononuclear cells in the lesions resulted in a reduced EAE score along with upregulated levels of anti-inflammatory cytokines such as IL-10 and IL-27 and expression of IDO. Further, there was reduced recruitment of Th1 and Th17 cells in the central nervous system following EnSC delivery. The results supported the promising idea of using EnSCs as a potent immunomodulatory tool for the treatment of neurodegenerative diseases.

10.7.4.3 Parkinson's Disease (PD)

PD is yet another important neurodegenerative concern, caused by the loss of dopaminergic neurons. EnSCs when transplanted into the striatum of a primate model of PD, resulted in successful engraftment, development of neuron-like morphology, expression of tyrosine hydroxylase (TH) and increased numbers of TH positive cells on the transplanted sites. Also, there were notable concentrations of dopamine metabolite in vivo [56].

10.7.5 Pancreatic Differentiation

EnSCs under specific induction conditions were able to differentiate to insulin producing pancreatic cells [51, 52]. They formed three-dimensional spheroid bodies (SBs) that secreted insulin and C-peptides in a glucose responsive manner similar to islet tissues. The differentiated SBs also expressed transcripts of NKx2.2, Glut2, insulin, glucagon, and somatostatin. A resistance to oxidative damage or oxidative damage-induced apoptosis was also observed with these SBs. When xenotransplanted into immunocompromised mice with streptozotocin-induced diabetes, these SBs were able to restore blood insulin levels [52]. Another study reported expression of pan β-cell markers and insulin secretion in pancreatic lineage differentiated EnSCs. Markers of pancreatic lineage such as PAX4, PDX1 and GLUT2 were increased in differentiated EnSCs compared to undifferentiated controls. When injected into the kidney capsules of diabetic mice, they promoted stabilisation of blood glucose within 5 weeks of administration [51].

10.7.6 Endometrial SCs: Contribution in Tissue Engineering

EnSCs have shown success in several utilities of tissue engineering. Ulrich et al. developed a tissue engineered scaffold using artificial meshes and tested its efficiency in a rat model of wound repair [64]. When grown and implanted onto meshes, EnSCs promoted neovascularization, exhibited reduced chronic inflammation, minimal fibrosis, increased tissue integration, deposition of collagen fibres and distensibility of the mesh. Additionally, there was no adverse foreign body reaction, thereby resulting in a successful implantation in the model. These biocompatible meshes were also suggested to be feasible for the treatment of pelvic organ prolapse (POP). Another major advancement in the utility of EnSCs in tissue engineering is their suggested use as a new cell source for reconstruction of urinary bladder tissue in women [65]. EnSCs were able to differentiate to urinary bladder smooth muscle cells in the presence of a hydrogel scaffold. The use of EnSCs has also been implicated in pancreatic tissue engineering. Niknamas et al. developed a fibrin hydrogel scaffold incorporating EnSC differentiated pancreatic beta cells. The EnSCs were able to form islet clusters and secreted insulin. These scaffolds further expressed markers of β cells like PDX1, proinsulin, and c-peptide [66]. Being an autologous source withless immunogenicity, EnSCs could be a safe tool for tissue engineering applications.

10.7.7 Other Therapeutic Benefits of EnSCs

Regenerative capacities of EnSCs were also established in certain other pre-clinical models such as of bone regeneration [57] and Glioma [67].

10.7.7.1 Bone Regeneration

EnSCs grown on biomimetic gelatin/apatite (Gel/Ap) scaffolds when implanted onto a critical size calvarial defect in the cranial bone of adult male rats resulted in significant bone formation and maturation [57]. The authors suggested the use of such biodegradable implants with good mechanical properties seeded with EnSCs as a therapeutic alternative for skeletal reconstructive surgery.

10.7.7.2 Glioma

The promise of EnSC therapy in managing disease models of Glioma has raised a paradoxical concern with respect to thieranti-angiogenic role in tumor tissues [67]. A study by Murphy et al. demonstrated the pro-angiogenic role of EnSCs when administered in a hind limb ischemia model [68]. Contrary to this finding, when administered into rat models of Glioma, EnSCs promoted significant inhibition in tumor angiogenesis. The number of CD 133 positive cells was also observed to be reduced in the tumor tissue supporting the tumor inhibitory activity of ERCs [67]. Further studies are however needed to determine the underlying mechanisms by which the administered EnSCs support an increased physiological angiogenesis as well as a reduced tumor angiogenesis.

10.8 EnSCs in Clinical Trials: Success Stories

In 2009, Zhong et al. described for the first time production of clinical grade 'Endometrial Regenerative Cells' (ERC) for treatment of multiple sclerosis [58]. Healthy non-smoking female volunteers aged 18–30 years served as endometrial donors in the study. A small clinical trial was performed wherein patients with multiple sclerosis were treated intravenously and intrathecally with menstrual blood-derived EnSCs by administering a series of 3–5 injections with a total dose of 16–30 million cells. The patients were followed for a longer time period, upto an year, for any immunological or adverse reactions. Results showed no immunological reactions or adverse side effects in all the four patients enrolled in the study suggesting the potential of EnSCs as a future therapeutic alternative for multiple sclerosis.

The second clinical trial using EnSCs was performed in a 23 year old male with Duchenne Muscular Dystrophy, a lethal X-linked musculo degenerative condition. The patient was treated with a dose of 116 million cells intramuscularly and followed up for 3 years. There was no adverse reaction reported following EnSC infusion and the patient was in general good health. Increased muscle strength and decreased respiratory infection was reported in the third year follow up [59, 69].

The third clinical report described utilization of EnSCs for ischemic cardiomyopathy [60]. The 74-year old patient, who participated in the study, received a combination of allogeneic CD34 cells and endometrial regenerative cells (ERC) at a total intravenous dose of 15 million cells over a period of one week. Patient was

followed up after an year and the promising findings of the study include no mass formation, inflammation or abnormalities at injection sites. Ejection fraction of the patient increased from 30% to 40% and there was a significant decrease in basic natriuretic peptide values. Further, radiological examination of the chest and lateral x-ray did not reveal any abnormalities. In the year 2012, Medistem Inc together with ERCell LLC initiated a clinical trial utilising endometrial regenerative cells (ERCs) for treating heart failure in a double blind, placebo controlled phase II trial [59]. The trial was successful in the preliminary round and is permitted to continue by the Data Safety Monitoring Board.

Though these reports showcase the potential benefits of endometrial stem cell mediated cardiac regeneration and its potential suitability to be pronounced as "off the shelf" biologically competent limitless sources of stem cells, the exact mechanism of cardiac regeneration is still unknown. Whether cardiac regeneration is the result of EnSCs undergoing differentiation to functional cardiac cells or executed via paracrine effects deliberated by secreted cytokines that activate survival pathways by recruiting endogenous progenitor stem cells is yet to be unravelled.

10.9 Pros and Cons of EnSC Therapy

Clinical trials have demonstrated the potential use of EnSCs in repairing damaged tissues and in correcting degenerative disorders without underlying immune complications and/or rejections. The ease of access, isolation and maintenance of EnSCs in culture for several generations and their diverse differentiation potential provide them an advantage for therapeutic usages. Certain non-invasive procedures are in place for obtaining menstrual blood for isolation of EnSCs using menstrual cups, which collect menstrual blood on day 2–3 of the menstrual period without any complications. Even though the techniques involved in harvesting EnSCs are standardised by a number of research groups, in terms of their clinical use, the existing procedures have a major disadvantage owing to contamination with cells other than stem cells majorly fibroblasts. In this context, it is important to note that, a significant portion of the current research on the plasticity of EnSCs was based on the cell population without purification. Lack of a specific identification system thus limits the setting of exact cGMP protocols in place for the production of EnSCs and emphasizes the need for further research in this area. Harvesting EnSCs from reproductive age women holds several advantages in comparison to MSC from other sources, mainly due to a greater ease of supply and the extended availability during a woman's lifetime with limited ethical concerns. The same, however, would not be possible in case of post-menopausal women and non-invasive techniques have to be used for harvesting EnSCs by obtaining endometrial biopsy samples. Furthermore, there is no information regarding the potency of EnSCs in post-menopausal women published in the literature.

Another major factor to consider for use of stem cells in therapy is their tendency for transdifferentiation, which is associated with a discrete change in the programme

of gene expression due to variation in the expression of master switch genes [70]. Transdifferentiation process is often associated with metaplasia and there are evidences in literature to support that circulating stem cells is a source of metaplastic transdifferentiation. In case of EnSCs, there also exists a theoretical risk for initiation of endometriosis following their in vivo delivery though there is no experimental evidence for the same. Also, almost all the in vivo reports regarding the use of EnSCs for regenerative therapy is in small laboratory animals, thus warrants further studies in larger animal models before their large scale use in clinical trials.

10.10 Conclusions

Recent years have witnessed a rapid growth with regard to unravelling the therapeutic possibilities of EnSCs in regenerative medicine. EnSCs were able to be harvested, characterised and their potency was studied using a variety of techniques from the uterine tissue. Comparative studies revealed that EnSCs have similar characteristic features as of bone marrow MSCs with respect to cytokine production, miRNAs and gene expression. EnSCs, however, possess higher proliferation potential and plausibly higher angiogenic potential compared to bone marrow MSCs although more studies are required to ascertain the later fact. Another important fact to note is their suggested protective role in acute inflammatory conditions and potent immunomodulatory effects.

Though the biological and clinical implications of EnSCs is a growing area of research, the field is relatively new and still not completely understood. There is definitely a need for more studies in higher animal models in some of the aspects where small laboratory animal studies have shown success. As with technological advancements these cells become better characterised, their role in gynaecological disorders associated with abnormal endometrial proliferation could also be delineated. This facilitates an increased knowledge in understanding disease pathogenesis of a number of pathological conditions such as endometrial cancer, endometrial hyperplasia, endometriosis and adenomyosis, thus enabling a suitable change in the way these diseases are treated in the future. Given the existing promising therapeutic benefits described in laboratory animal studies and in a few clinical trials, we envisage that EnSCs could offer considerable assistance for the future of regenerative medicine.

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