Stem Cells in Clinical Applications

Phuc Van Pham *Editor*

Neurological Regeneration

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Stem Cells in Clinical Applications

Series editor
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Stem Cells in Clinical Applications brings some of the field's most renowned scientists and clinicians together with emerging talents and disseminates their cutting-edge clinical research to help shape future therapies. While each book tends to focus on regenerative medicine for a certain organ or system (e.g. Liver, Lung and Heart; Brain and Spinal Cord, etc.) each volume also deals with topics like the safety of stem cell transplantation, evidence for clinical applications including effects and side effects, guidelines for clinical stem cell manipulation and much more. Volumes will also discuss mesenchymal stem cell transplantation in autoimmune disease treatment, stem cell gene therapy in pre-clinical and clinical contexts, clinical use of stem cells in neurological degenerative disease, and best practices for manufacturers in stem cell production. Later volumes will be devoted to Safety, Ethics and Regulations, Stem Cell Banking and Treatment of Cancer and Genetic Disease.This series provides insight not only into novel research in stem cells but also their clinical and real-world contexts. Each book in Stem Cells in Clinical Applications is an invaluable resource for advanced undergraduate students, graduate students, researchers and clinicians in Stem Cells, Tissue Engineering, Biomedical Engineering or Regenerative Medicine.

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Preface

Neurological diseases are disorders in the central nervous system or the peripheral nervous system that is caused by structural, biochemical, and electrophysiological dysfunctions or abnormal functions of neurons or glial cells. There are some groups of neurological diseases including neurodegenerative diseases such as Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis, and others related to dysfunctional blood circulation such as ischemic and hemorrhagic strokes or cancer such as glioma.

Although there are different symptoms, all neurological diseases are results from the significant loss of neurons or glial cells. The regeneration of these cells is considered as the promising strategy to treat diseases. Stem cell therapy draws attention as a promising regenerative option for the treatment of various neurologic diseases.

In recent years, stem cell therapies are moved to the clinic with exciting results. There are at least three ways that stem cells can correct injured neurological tissues including cell replacement, paracrine factors, and immunomodulation. Indeed, some kinds of stem cells, such as embryonic stem cells, induced pluripotent stem cells, neural stem cells, or mesenchymal stem cells, are easily differentiated or trans-differentiated into neural cells. Transplantation of these stem cells or differentiated cells from these stem cells can supply new neural cells to regenerate injured or degenerative tissues. In addition to direct cell replacement, stem cells can secrete various cytokines and growth factors that elicit a variety of beneficial effects such as neural cell protection and induction of the endogenic recovery system. Recently, mesenchymal stem cells are proved as effective immunomodulatory factors. By direct communication or via cytokines, mesenchymal stem cells can suppress the inflammatory process, inhibit the autoimmune reactions, etc.

This volume of *Stem Cells in Clinical Applications* book series entitled *Neurological Regeneration* aims to provide updated invaluable resource for advanced undergraduate students, graduate students, researchers, and clinicians in stem cell applications for neurological regeneration.

This book with 13 chapters covers almost the present applications of stem cells in the central and peripheral nervous system regeneration. Chapter 1 introduces and

updates recent applications of stem cells in neurological regeneration. Chapters 2, 3, 4, 5, 6, 7, 8, and 9 introduce some recent approaches of stem cells in brain regeneration, and spinal injury healing, respectively. And Chaps. 10, 11, 12, and 13 focus on peripheral nervous system regeneration including tympanic membrane, retina, and cornea.

We are indebted to our authors who graciously accepted their assignments, and who have infused the text with their energetic contributions. We are incredibly thankful to responsible editor Aleta Kalkstein, and the staff of Springer Science + Business Media that published this book.

Ho Chi Minh City, Vietnam Phuc Van Pham

Contents

Biography

Phuc Van Pham received his Ph.D. in Human Physiology from Vietnam National University, Ho Chi Minh City, Vietnam. He is currently a Professor of Biology at Vietnam National University and Director of the Laboratory of Stem Cell Research and Application. He is a longstanding lecturer and translational scientist at the University and is a member of several societies and journal editorial boards focused on stem cells.

Dr. Pham and his colleagues have established one of the first multidisciplinary stem cell centers in Vietnam, and he has successfully launched an array of technologies in stem cell isolations. His research interests include stem cell isolation, stem cell therapy, mesenchymal stem cells, cancer stem cells, immunotherapy, and regenerative medicine, and he has published extensively in these areas.

After many years of experience as an embryologist, cell biologist, and molecular biologist, collaborating with leading researchers in Singapore, Japan, and the United States, Dr. Pham is a student again, keen to reach beyond the traditional boundaries of biology.

Chapter 1
Stem Cell Therapy in Neurological and Neurodegenerative Disease

Hong J. Lee, Sung S. Choi, Sang-Rae Lee, and Kyu-Tae Chang

1.1 Introduction

Stem cells are self-renewing and pluripotent, meaning that they are capable of continuous proliferation and terminal differentiation into various cell types. Stem cells are classified as either embryonic or adult based on their origin, and give rise to various organs and tissues (Thomson et al. 1998; Shamblott et al. 1998). Recent studies indicated that induced pluripotent stem cells (iPSCs) and directly induced neurons are included in the category of stem cells (Takahashi and Yamanaka 2006; Pang et al. 2011).

Stem cell transplantation has enabled powerful new therapeutic strategies in research for the treatment of various human neurological diseases such as Alzheimer's disease (AD) and Huntington's disease (HD) (Kim et al. 2013; Nikolic et al. 2008). The identification, generation, and optimization of suitable stem cell types for cell therapy is necessary for the full utilization of this promising therapeutic approach in neurological disease (Kim et al. 2013). In this chapter, we review the utility and limitations of different stem cell types and discuss recent advances in the therapeutic use of stem cells in neurological and neurodegenerative disease.

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1.2 Types of Stem Cells

Stem cells are undifferentiated cells that are capable of symmetrical and/or asymmetrical division by mitosis, and are also able to differentiate into mature tissue-specific cells (Gage and Temple 2013; Martello and Smith 2014; Golas and Sander 2016). Stem cells have been used for the treatment of various neurological diseases including AD and HD; cells for these purposes have been derived from embryonic stem cells (ESCs), induced pluripotent stem cells, and tissue-specific adult stem cells (Hargus et al. 2014; Maucksch et al. 2013; Schwarz and Schwarz 2010).

1.2.1 Embryonic Stem Cells

Human ESCs (hESCs) are pluripotent cells derived from the inner cell mass (ICM) of the blastocyst. hESCs can differentiate into all cell types from three embryonic germ layers: the endoderm, mesoderm and ectoderm (Thomson et al. 1998). Accordingly, these cells can be applied therapeutically in human disease states (Nishio et al. 2016). Although hESCs initially require culture on a feeder layer of mouse embryonic fibroblasts and animal serum (Thomson et al. 1998), many studies have established animal substance-free, feeder-free hESC culture conditions for clinical applications (Hovatta et al. 2014). Because hESCs are derived from *in vitro* fertilized blastocysts donated for research, the transplantation of hESCs faces ethical and legal limitations (Hovatta et al. 2010; Lo et al. 2005).

1.2.2 Induced Pluripotent Stem Cells

Takahashi and Yamanaka were the first to reprogram mouse embryonic and adult fibroblasts into iPSCs by transduction with retroviral vectors carrying genes encoding the transcription factors Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka 2006; Takahashi et al. 2007). Adult human fibroblasts have also been reprogrammed into iPSCs by transduction with a lentiviral vector carrying *OCT3/4*, *SOX2*, *NANOG*, and *LIN28* (Yu et al. 2007). iPSCs have properties that grant them similar a therapeutic potential to that of hESCs, such as self-renewal and the ability to differentiate into any cell lineage (Lowenthal et al. 2012). However, the use of viral vectors always carries the risk of off-target viral gene insertion in the genome and thus many researchers have made efforts to develop more specific vectors such as the Sendai virus (Fusaki et al. 2009) as well as vector-free transfection methods (Warren et al. 2010). In addition optimizing the reprogramming procedure, the potential risk of teratoma formation continues to be an obstacle for the therapeutic use of iPSCs.

1.2.3 Tissue-Specific Stem Cells

Tissue-specific stem cells are intrinsic cell lineages derived from specific tissues; examples include neural stem cells (NSCs) (De Filippis and Binda 2012) and mesenchymal stem cells (MSCs) (Bantubungi et al. 2008). Tissue-specific stem cells are in current use for the treatment of neurodegenerative disease (Jiang et al. 2011).

NSCs are initially capable of differentiating into one of three brain cell lineages: neurons, astrocytes, and oligodendrocytes (Eriksson et al. 1998). NSCs can be isolated from neurogenic regions of the brain, which include the subventricular zone of the lateral ventricle and the subgranular zone of the hippocampus (Bernier et al. 2000; Lois and Alvarez-Buylla 1993; Eriksson et al. 1998). However, these cells can only be isolated from aborted fetal tissue (Monni et al. 2014; Lee et al. 2007), tissues excised during neurosurgery (Sanai et al. 2004), and postmortem tissues (van Strien et al. 2014), such that the use of NSCs is accompanied by practical and ethical limitations. Alternatively, NSCs have been immortalized using viral vectors carrying oncogenes such as v-myc (Lee et al. 2007) to address the limited self-renewal capacity of non-immortalized NSCs (Yang et al. 2003).

MSCs were firstly isolated from bone marrow in 1976 (Friedenstein et al. 1976). Human MSCs are multipotent cells with long-term self-renewal that can differentiate into mesenchymal tissues such as bone, cartilage, fat, tendon, muscle, and marrow stroma (Pittenger et al. 1999). MSCs can be isolated from various tissues including bone marrow, adipose tissue, placenta, umbilical cord blood, lung, and amniotic fluid (de Ugarte et al. 2003; Campagnoli et al. 2001; In't Anker et al. 2004; Noort et al. 2002; Fan et al. 2005; Tsai et al. 2004). The accessibility and flexibility of MSCs has made them a suitable candidate for therapeutic use (Hashemian et al. 2015).

1.3 Stem Cell Therapy for Neurological Diseases

1.3.1 Alzheimer's Disease

Neurons differentiated from transplanted stem cells have been demonstrated to integrate into the host brain and improve learning and memory (Liu et al. 2013). In another study, transplantation of stem cells overexpressing choline acetyltransferase led to increased brain acetylcholine and improved cognitive performance in a murine model of AD (Park et al. 2013a). Furthermore, stem cells expressing neurotrophic factors such as nerve growth factor have been demonstrated to differentiate into neurons and restore cognition (Lee et al. 2012, Fig. 1.1) as well as modulate neurogenesis (Park et al. 2013b) in murine AD.

Neural stem cells differentiated from ESCs have been employed in a rodent model of AD: transplanted cells were successfully differentiated into neurons and

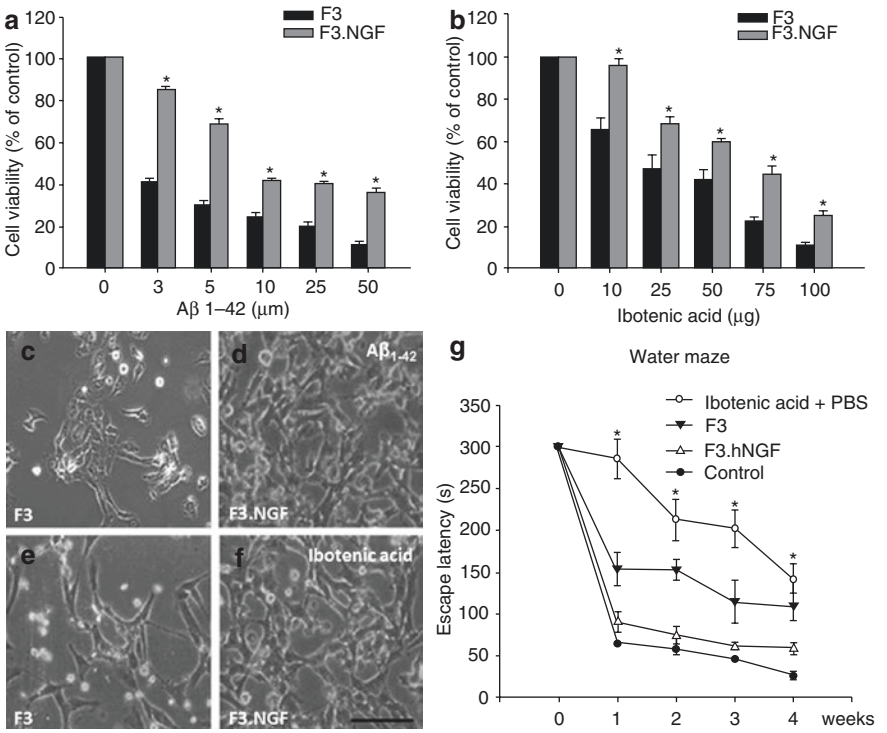

Fig. 1.1 The neuroprotective and therapeutic effects of neural stem cells for AD. (**a**, **b**) Cell viability of F3 and F3.NGF under various concentration of Aβ and ibotenic acid. F3.NGF cells showed much higher cell survival compare to F3 (control). (**c**, **d**) Phase contrast microscopy images of F3 and F3.NGF in the presence of 5 μM Aβ (**c**, **d**) and 25 ug/ml ibotenic acid (**e**, **f**). (**g**) Learning and memory improvement improved in F3.NGF transplanted mice (Lee et al. 2012)

astrocytes, and led to improved cognitive function as measured by the Morris water maze test (Tang et al. 2008). Alternatively, iPSCs have been investigated in the context of early-onset familial AD; in one study, iPSCs differentiated into neurons secreting $A\beta_{1-42}$ were sensitive to γ-secretase inhibition (Yagi et al. 2011), which demonstrated the validity of these cells as an *in vitro* model for AD. While this application was not directly therapeutic, the results suggest that these iPSCs provide an innovative strategy for drug research and development for AD. Finally, MSCs are perhaps the most well researched stem cells in the context of AD. In a rodent model of AD, transplantation of MSCs from bone marrow reduced the number of Aβ deposits in the hippocampus by producing endogenous microglial activation (Salem et al. 2014; Lee et al. 2009). Human MSCs have also been shown to significantly enhance the autophagic system and promote Aβ clearance and thus neuronal survival in an Aβ-treated mouse model (Shin et al. 2014). In a murine aging, the transplantation of adipose-derived stem cells was identified to enhance brain acetylcholine, modulate microglial activation, and improve cognitive function (Park et al. 2013b; Ma et al. 2013). The therapeutic application of MSCs was recently advanced by the development of NEUROSTEM®-AD human umbilical cord blood/derived MSCs by Medipost co. LTD. In one study, co-culturing of these cells with microglia led to

the supernatant elevations in ICAM-1 and upregulation of the neprilysin, an Aβ-degrading enzyme, in microglia (Kim et al. 2012). At present NEUROSTEM®-AD cells are in Phase I/II clinical trials for AD. Taken together, these data suggest that stem cells have therapeutic utility in the context of AD.

1.3.2 Parkinson's Disease

PD is one of most common aging-related neurodegenerative and neurobehavioral disorders, and is characterized by the loss of dopaminergic neurons in the substantia nigra. Histologically, PD is typified by Lewy body deposits that contain alpha-synuclein and ubiquitin (Beitz 2014; Kim et al. 2013). Administration of L-dihydroxyphenyl alanine (L-DOPA) is an effective treatment for PD; however, L-DOPA is associated with side effects that complicate its long-term use (Kim et al. 2013). Stem cell therapy may thus be a useful alternative for improving PD symptoms.

In one study, functional deficits associated with neurological disease were attenuated via transplantation of NSCs secreting specific neurotransmitters and neurotrophic factors. Striatal transplantation of the HB1.F3 NSC line, which is immortalized by a retroviral vector carrying *v-myc*, produced neuroprotection against dopaminergic cell depletion in a rodent model of PD. Some studies have also reported that ESC-derived neurons have therapeutic effects in PD: ESC-derived dopaminergic neurons were able to recover behavior deficits in rodent PD (Cho et al. 2008; Chung et al. 2011; Kriks et al. 2011). iPSCs have additionally demonstrated utility in PD. Dopaminergic neurons can be differentiated from iPSCs and have the properties of mature neurons as measured by electrophysiological and morphological analyses. Transplantation of these cells improved symptoms in a rodent model of PD (Wernig et al. 2008). In another study, iPSCs were differentiated into functional dopamine neurons by protein-based reprogramming and were able to rescued motor deficits following transplantation in rodent PD (Rhee et al. 2011). iPSCs may provide an ideal therapeutic cellular source for PD because they escape the problem of immune rejection. Alternatively, human parthenogenetic neural stem cells (hpNSC) derived from unfertilized oocytes escape ethical problems and represent an unlimited cellular supply. When these cells were transplanted into rodent and primate models of PD, they were engrafted successfully and produced higher levels of dopamine in the striatum without any adverse events (Gonzalez et al. 2015). Currently, human parthenogenetic neural stem cells (hpNSC) are in Phase I clinical trials for PD.

1.3.3 Huntington's Disease

HD is an autosomal and age-dependent neurodegenerative disorder that is characterized by abnormalities of movement, cognitive impairment, and emotional disturbances. HD is caused by the abnormal expansion of a CAG repeat in the huntingtin gene (Kim et al. 2008; Cattaneo et al. 2005).

In one study, human fetal striatal tissue containing neurons and glia was transplanted into the neostriatum of an HD patient, successfully integrated without immune rejection, and grown to a size of 2.9 cm. Unfortunately, this study reported that fetal neural grafts have the potential risk of graft outgrowth after transplantation (Keene et al. 2009). Alternatively, transplant of 3–4 embryonic tissues into the bilateral caudate nucleus compensated for degenerated striatal tissue and improved cognitive symptoms in patient (Sramka et al. 1992). Recently, one group reported improved survival and the attenuation of cognitive decline in 10 patients with HD following fetal striatal graft implantation; these patients showed increases in striatal/cortical metabolism 2 years post-transplantation and less pronounced motor/cognitive decline (Paganini et al. 2014). However, most fetal striatal tissues for use in clinical trials have been derived from elective or medically warranted abortions. Thus, human fetal tissue transplantation is associated with significant ethical limitations.

1.3.4 Cancer

One therapeutic strategy of for cancer is the stem cell-based gene therapy as therapeutic gene carrier. Stem cells have potential to migrate into disseminated tumor in several studies (Shimato et al. 2007; Joo et al. 2009). And stem cells can deliver the enzyme-encoding gene to tumor cells and prodrug is locally converted into active cytotoxic chemical which kill the enzyme-producing cells as well as surrounding cells including tumor cells (Lim et al. 2011).

Yeast cytosine deaminase (yCD) gene was used to treat cancer in lung cancer brain metastases model. yCD gene can convert the prodrug, 5-fluorocytosine (5-FC) into cytotoxic 5-fluorouracil (5-FU). Human neural stem cells were genetically engineered with yCD carrying retrovirus and examined the therapeutic efficacy for lung cancer in presence 5-FC. These cells could remove the most of brain (Yi et al. 2012; Aboody et al. 2013) and prostate cancer (Lee et al. 2013) in vitro and in vivo (Fig. 1.2).

In addition, the combination of CD and herpes simplex virus thymidine kinase (TK), converting monophosphorylate ganciclovir to toxic triphosphate ganciclovir, gene in human amniotic fluid-derived stem cells (AF2.CD-TK) are also localized at eh tumor site and have potent cytotoxicity in presence of prodrugs (Kang et al. 2012).

HB1.F3 NSCs were first derived from human fetal brain at 8–18 weeks of gestation (Lee et al. 2012). In one study, HB1.F3 NSCs were transduced with a retrovirus carrying the gene for carboxyl esterase as suicide gene, and the resultant cell line was termed F3.rCE. Carboxyl esterase can convert irinotecan, a prodrug, into a bioactive toxic agent known as SN-38. F3.rCE cells were transplanted into medullobalstoma model (Lim et al. 2011) and brain metastasis model of breast cancer (Seol

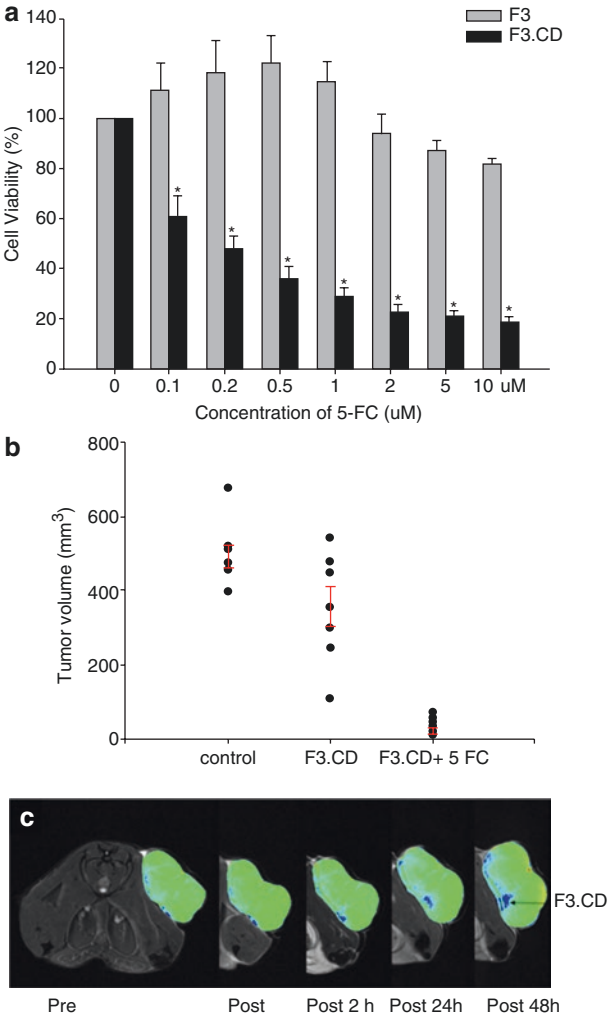

Fig. 1.2 F3.CD have the therapeutic effect for prostate cancer. (**a**) Suicide effect of F3.CD/5-FC system at 72 h after 5-FC treatment in vitro. (**b**) Efficient reduction of prostate tumor size observed in combination of F3.CD and systemic administration of 5-FC in vivo. (**c**) Transplanted F3.CD cells (*Blue*) (*arrow*) migrated inside the prostate cancer (*Green*) (Lee et al. 2013)

et al. 2011). F3.rCE showed the efficient therapeutic effects for brain and breast cancer in vitro and in vivo. F3.rCE cells have demonstrated both tumor tropism and the ability to produce effective SN-38 toxicity in proximal tumor cells. These cells are currently in a Phase I clinical trial for the treatment of glioma (Gutova et al. 2013).

1.4 Conclusion

Neuronal cell loss followed by the breakdown of tissue homeostasis is a hallmark of neurological disease. Thus, the induction of neurogenesis and the maintenance of homeostasis are important therapeutic goals in these patients. Regenerated neurons and glia can produce an optimal microenvironment for cell survival and regeneration. Transplanted stem cells can also be a source of neuroprotective molecules such as neurotrophic factors that assist neural cell survival after transplantation. Stem cells can also be excellent genetic carriers by retroviral and lentiviral transduction. Given what is known regarding neurological diseases, gene-carrying stem cell therapy systems have the potential to improve pathological disease processes. Moreover, iPSCs are an unlimited source of stem cells applicable as autologous cell therapy. It was recently reported that age-related mitochondrial DNA mutations can lead to respiratory defects in iPSCs from old people and accumulated mitochondrial DNA mutation can limit therapeutic potential of iPSCs (Kang et al. 2016). Further studies are also needed not only to examine the quality of transplanted stem cells over time but also to determine the optimal conditions for the recovery of damaged brain with stem cells in clinical trials.

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Chapter 2
Stem Cell Therapy in Traumatic Brain Injury

Sicong Tu and Jian Tu

2.1 Introduction

Traumatic brain injury remains a leading cause of mortality and long-term disability worldwide. Traumatic brain injury results in enormous losses to individuals, families, and communities (Corrigan et al. 2010). World Health Organization has estimated that 25 % of road traffic collisions requiring admission to a hospital suffered traumatic brain injury in 2004 (Corrigan et al. 2010; Atlas: country resources for neurological disorders home page; Global burden of disease estimates). World Health Organization has also introduced the new metric tool – the disability adjusted life year, which quantifies the burden of diseases, injuries and risk factors. The worldwide leading causes of traumatic brain injury include road traffic accidents that were estimated being 41.2 million disability adjusted life year in 2008, violence being responsible for 21.7 million disability adjusted life year, and self-inflicted injuries being 19.6 million disability adjusted life year, respectively. All these will leave disability associated with traumatic brain injury in survivors (Atlas: country resources for neurological disorders home page; Global burden of disease estimates).

However, no effective therapy or program is available for treatment of individuals with traumatic brain injury; nonetheless, researchers had tried some therapeutic agents like levodopa/carbidopa and some neurotrophic factors in brain injury with persistent vegetative state with the aim of augmenting and slowing the progression from persistent vegetative state into some degree of consciousness.

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This still needs experimentation to confirm if these dopamine precursors and other neurotrophic factors have any role in traumatic brain injury. Several other therapeutic agents like carnabinoid dexanabinol, erythropoietin, and gamma-glutamylcysteine ethyl ester have all shown to have neuroprotective effect in human at experimental stage with remarkable improvement in post-traumatic brain injury outcome (Nori et al. 2012; Ugoya and Akinyemi 2010; Biegon 2004; Lok et al. 2011; Maas 2001).

Recent advancement in knowledge about stem cells promotes the translation of stem cells to therapy in traumatic brain injury. The stem cells may play an important role in the treatment of traumatic brain injury by replacing damaged cells, and helping long-term functional recovery. The search for stem cell therapy for traumatic brain injury is promising and progressing. One obstacle in the search for an effective stem cell therapy is that the pathophysiology of traumatic brain injury is largely unknown. This is because multiple cell types like neuronal cells, glial, and endothelial cells are usually involved in traumatic brain injury. Furthermore, cerebral vasculature, especially the blood brain barrier may be affected in traumatic brain injury; this injury may be focal or diffuse axonal injury. Taming these burgeoning effects of traumatic brain injury requires neural stem cells which can differentiate into neurons and glial cells. It has been reported that progenitor cells differentiated into neurons and glial in adult brain, and an increase in astrocytic progeny forming reactive astrocytes to primarily limit cyst enlargement in posttraumatic syringomyelia (Mammis et al. 2009; Stoica et al. 2009; Tu et al. 2010, 2011).

This chapter is an optional extra to confirm whether we can achieve the translation of basic knowledge of neural stem cells into therapeutic options in persons with traumatic brain injury by enhancing and integrating these neural precursor cells unto neurogenesis and directing these cells to the specified targets or through multipotency where the transplanted stem cells can differentiate into glial cells, neurons and endothelial cells. As traumatic brain injuries are not always focal but diffuse we need to induce these transplanted stem cells differentiating into appropriate phenotype for long term structural and functional recovery. This chapter critically reviews current literatures on neural stem cell research and proposing an approach for quality clinical translation of stem cell research to therapy in traumatic brain injury. The author explains the pathophysiology of traumatic brain injury and proposes the "six point schematic approach" to achieving quality bench to bedside translation of neural stem cells to therapy for traumatic brain injury. The author also highlights the need for suitable clinical translation, coordination, and administration of research in the field of stem cell therapy for traumatic brain injury.

2.2 Neuropathology of Traumatic Brain Injury

Neuropathology of traumatic brain injury involves two main phases. These are the primary brain injury following the trauma, and the secondary injury which are mediated by inflammatory response to the primary brain injury.

2.2.1 *Primary Injury After Traumatic Brain Injury*

Neuropathology of the initial brain injury has been postulated to include acceleration, deceleration, and rotational forces which may or may not be as a result of the trauma. This sequence of events leads to the initiation of inertia which is both acceleration and rotational head movement. This impact on the cortical and sub-cortical brain structures causes focal or diffuse axonal injury and these inertial forces disrupt the blood brain barrier (Albert-Weissenberger et al. 2012). The primary events also involve massive ionic influx referred to as traumatic depolarization. The major inflammatory neurotransmitters released from the damaged tissue are excitatory amino acids, which may explain the neuropathology of diffuse axonal injury in traumatic brain injury. This is followed by cerebral edema with associated increase in intracranial pressure, usually forms the major immediate consequences of traumatic brain injury. Brain edema may come from astrocyte swelling and disruption of the blood brain barrier (Povlishock 1992; Greve and Zink 2009). The blood brain barrier is disrupted in acute phase of severe traumatic brain injury. The expression of high levels of glucose transporter 1 was observed in capillaries from acutely injured brain, which occurs in association with compromised blood brain barrier function. Vascular endothelial growth factor also plays a role in neuronal tissue disruption and increases the permeability of the blood brain barrier via the synthesis and release of nitric oxide. Figure 2.1 depicts the neuropathology of the primary injury after traumatic brain injury.

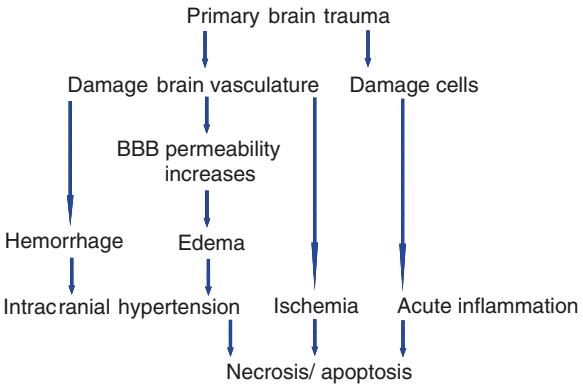

Fig. 2.1 Sequential events of the primary injury in traumatic brain injury. Initial impact is usually by direct trauma to the head either open or closed head injury. This trauma causes mechanical damage to neurons, axons, glia, and blood vessels by shearing, tearing or stretching. Blood vessel ruptures cause hemorrhage. Even in unruptured blood vessels, the permeability of blood brain barrier increases resulting in edema. Hemorrhage and edema often lead to intracranial hypertension. Following hemorrhage, ischemia could occur in brain tissue. Traumatic brain injury caused cell damage induces macrophage and lymphocytes migrant to the injury site releasing inflammatory mediators that triggers a cascade of events towards necrosis and/or apoptosis. Necrosis and/or apoptosis also can be a consequence of hemorrhage and ischemia

2.2.2 *Secondary Injury After Traumatic Brain Injury*

The secondary events are a complex association of the inflammatory response initiated by the trauma leading to diffuse neuronal degeneration of neurons, glial, axonal tearing, and genetic predisposition (Fig. 2.2). Furthermore, excitatory amino acids release, oxygen radical reactions, and nitric oxide production lead to the activation of N-methyl-D-aspartate, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid, alpha-7 nicotinic receptor (α7), and nicotinic acetylcholine receptor (Hinzman et al. 2012; Goforth et al. 2009; Kelso and Oestreich 2012) and subsequent calcium influx. All these cascades of events cause mitochondrial disruption and free radical release with eventual tissue peroxidation. One theory is that excitatory amino acid release leads to calcium influx into neurons and other brain cells which promote oxygen free radical reactions. High calcium and the presence of free-radical molecules create an unstable environment in the brain cells that lead to increased

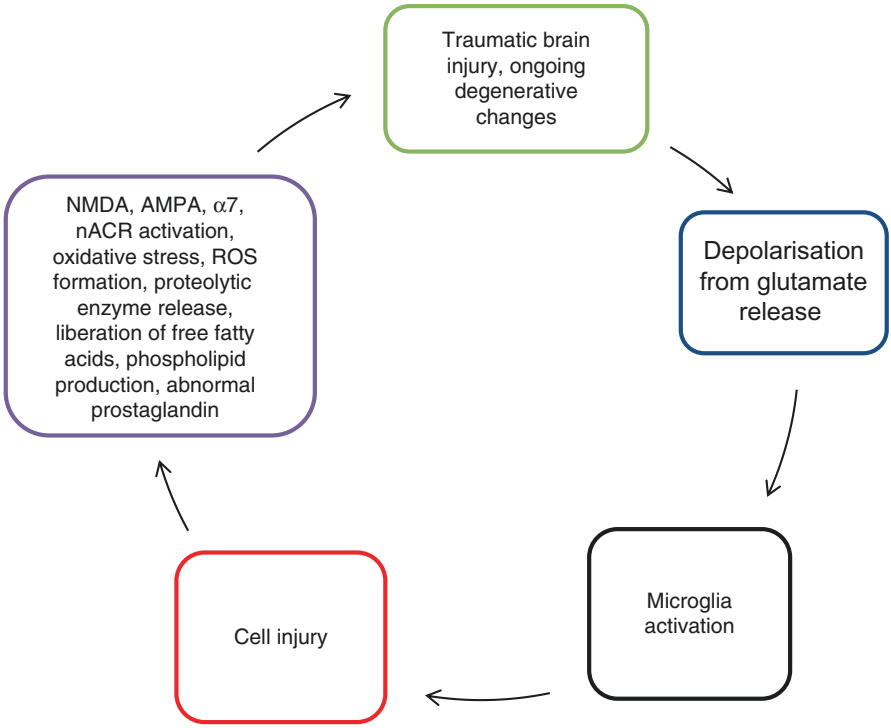

Fig. 2.2 Sequential events of the secondary injury in traumatic brain injury. This includes variety of processes, such as depolarization, disruption of ionic homeostasis and release of neurotransmitters, lipid degradation, and oxidative stress. These events are a result of interaction between the excitatory amino acids released with an influx of oxygen free radicals that ultimately set up N-methyl-D-aspartate, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid, α7 and nicotinic acetylcholine receptor to sustain the unstable environment for cell injury and degenerative changes

production and release of nitric oxide and excitatory amino acids, such as glutamate. Nitric oxide participates in oxygen radical reactions and lipid peroxidation in neighboring cells (Stoffel et al. 2001). A summary of the secondary injury after traumatic brain injury is shown in Fig. 2.2. The secondary injury plays a major role in the outcome of traumatic brain injury. Therapeutic interventions should target this phase as it is the major determinant of morbidity and mortality in traumatic brain injury (Nawashiro et al. 1994). Clinically, the application of stem cell therapy early to patients with traumatic brain injury is ethically challenging because of the difficulty in obtaining informed consent immediately following the brain injury. Genes implicated to influence the outcome of traumatic brain injury include *apoe*, *comt*, *drd2*, *ace*, and *cacna1a. Apoe* multifactorially affects the clinicopathological consequences of traumatic brain injury (Potapov et al. 2010). *Apoe* is associated with increased amyloid deposition, amyloid angiopathy, larger intracranial hematomas, and more severe contusional injury. *Comt* and *drd2* are genes which influence dopamine-dependent cognitive and behavioral processes, such as executive or frontal lobe functions. The *ace* gene affects traumatic brain injury outcome *via* alteration of cerebral blood flow and/or autoregulation. The *cacna1a* gene exerts an influence *via* the calcium channel pathways and its effect on delayed cerebral edema (Jordan 2007). Increased signal transducers and activator of transcription 3 signaling has been reported in a rodent model of traumatic brain injury (Oliva et al. 2012). Although several potential genes that may influence the outcomes following traumatic brain injury have been identified, future investigations are needed to validate these genetic studies, and identify new genes that might contribute to the patient outcomes after traumatic brain injury.

2.3 Current Pharmacotherapy for Traumatic Brain Injury

Pharmacotherapies aim at promoting neurorepair, neuroregeneration, and neuroprotection following traumatic brain injury. Clinical trials evaluating these interventions apply standardized clinical outcome measures to demonstrate efficacy. In the past, drug research and development for traumatic brain injury focused on limiting secondary brain injury after the initial traumatic event because of lacking evidence that the central nervous system could be repaired or regenerated. Growing body of evidence indicates that the adult brain can be repaired and regenerated after traumatic brain injury. Potential drug targets for post-traumatic injury brain repair include angiogenesis, axon guidance and remodeling, remyelination, neurogenesis, and synaptogenesis. Pharmacotherapies may also target brain regeneration by enhancing the capacity of pluripotent cells to differentiate into neurons, glia, and vascular endothelium (Jin et al. 2011; Valable et al. 2010; Xiong et al. 2008a, 2010a; Yatsiv et al. 2005; Zhang et al. 2009, 2010). Brain repair and regeneration processes can be activated or enhanced by pharmacotherapy over a longer therapeutic window than pharmacologic interventions designed to limit injury. Pharmacotherapies are potentially effective in the acute, subacute, post-acute, and chronic phases after

traumatic brain injury. Thus, repair and regeneration therapies have the potential advantage of being effective over a prolonged period of time following traumatic brain injury.

Currently, no effective pharmacologic agent has received approval from the U.S. Food and Drug Administration for the treatment of patients with traumatic brain injury. Table 2.1 lists candidate compounds currently undergoing clinical evaluation for traumatic brain injury treatment. Because traumatic brain injury damages the brain tissue by multiple mechanisms, combination therapy designed to simultaneously target multiple mechanisms of injury is likely required. To date, all phase II/III traumatic brain injury clinical trials have failed (Xiong et al. 2012; Watanabe et al. 2013). Stem cell therapy offers an alternative option for traumatic brain injury.

2.4 Stem Cell Therapy in Traumatic Brain Injury

There are at least two strategies involving stem cell therapy to repair injured brain tissue. They are transplantation of exogenous stem cells to replace damaged cells and stimulation of endogenous stem cells to proliferate to the number of cells needed and differentiate them to the phenotype of cells required for normalization of brain function.

2.4.1 Transplantation of Exogenous Stem Cells in Traumatic Brain Injury

There is great number of attempts to transplant various types of cells, such as neurons, and neural stem cells to repair damaged brain tissue. The main objectives of transplantation experiments are (1) growth facilitation: the transplant fills the lesion site and serves as a cellular bridge; (2) new neurons: the transplant can provide new neurons, which in turn provide new targets and sources of innervations and thus repair the damaged neural circuits; (3) factor secretion: the transplant can produce a variety of substances, such as neurotrophic factors, that promote the brain tissue repair process (Barami and Diaz 2000). Several characteristics of neural stem cells make them potentially suitable to repair damaged brain tissue after traumatic brain injury. Firstly, they can serve as a renewable supply of transplantable cells by clonal expansion in cell culture. Secondly, they are of central nervous system origin and the stem cells generated from the grafts have neural characteristics. Thirdly, neural stem cells can be manipulated by genetic engineering methods to produce specific proteins, such as neurotrophins, neurotransmitters and enzymes (Pincus et al. 1998).

It has been reported that autologous cultured cells harvested at time of emergency surgery from patients with traumatic brain injury, and subsequently engrafted into damaged part of the brain can be detected using magnetic resonance imaging

Table 2.1 Pharmacotherapies currently undergoing clinical evaluation for traumatic brain injury treatment

Compound	Neuroprotective mechanism	Preclinical evidence	Clinical evidence	References
Progesterone	Enhances behavioral and functional outcomes, myelination and neurogenesis Decreases brain edema, apoptosis, pro-inflammatory cytokines Inhibits gamma-aminobutyric acid receptors Prevents neuronal cell death	Reduces IL-6, IL-1β, TNF-α, mitochondrial dysfunction, neuronal loss, axonal injury, tissue loss, lesion size, intracranial pressure, astrocytic accumulation Inhibits neuronal calcium signalling, Toll-like receptor signalling Modulates aquaporin 4 expression, NFk-B signalling, cell proliferation Enhances CD55 production, superoxide dismutase activity, levels of neurotrophin factors Protects against lipid peroxidation Improves spatial learning and memory, locomotor activity and outcomes, anxiety-like behaviors, motor and cognitive performance	Ongoing Phase III clinical trials are positive	Atif et al. (2013), Abdel Baki et al. (2010), Hammond et al. (1983), Lacroix et al. (1987), Lanthier and Patwardhan (1986), Weill-Engerer et al. (2002), Koenig et al. (1995), Liu et al. (2009), Porcu et al. (2009), Sayeed et al. (2006, 2009), Hua et al. (2011, 2012), Cutler et al. (2006, 2007), Galani et al. (2001), Grossman et al. (2004, 2011), Shahrokhi et al. (2010, 2012), Kasturi and Stein (2009), Pan et al. (2007), Guo et al. (2006), Robertson et al. (2006), Roof et al. (1992, 1994, 1996, 1997), Cekic et al. (2012), Chen et al. (2007a, 2008a, b), O'Connor et al. (2007), Yao et al. (2005), Djebaili et al. (2004, 2005), He et al. (2004), Sarkaki et al. (2013), VanLandingham et al. (2007), Jones et al. (2005), Peterson et al. (2012), Luoma et al. (2011, 2012), Garcia-Estrada et al. (1993, 1999), Pettus et al. (2005), Barha et al. (2011), Anderson et al. (2011), Wali et al. (2011), Gilmer et al. (2008), Wright et al. (2007), Xiao et al. (2008), Benvenga et al. (2000)
Growth hormone	Normalize growth hormone deficiency/insufficiency occurred as a result of direct pituitary or indirect hypothalamic injury in 20 % TBI patients Has neuroprotective and neuroregenerative effects	Improves motor function, spatial learning and memory Enhances learning and memory retention Corrects impairments of endothelial progenitor cells Has direct autocrine and/or paracrine neuroprotective effects, anti-apoptotic effect, wound healing effects	Cognitive improvement in growth hormone deficiency/insufficiency TBI patients FDA-approved for adult patients with acquired growth hormone deficiency	Benvenga et al. (2000), Berg et al. (2010), Kelly et al. (2000), Lieberman et al. (2001), Urban (2006), Wilkinson et al. (2012), Giordano et al. (2005), Saatman et al. (1997), Doulah et al. (2009), Svensson et al. (2006), Thum et al. (2007), Creyghton et al. (2004), Ling et al. (2007), Barlind et al. (2010), Sanders et al. (2008, 2010), Demling (1999, 2005), Herndon et al. (1990), Luo et al. (2000), Devesa et al. (2013), High et al. (2010), Reimunde et al. (2011), Takala et al. (1999)

(continued)

Table 2.1 (continued)

Compound	Neuroprotective mechanism	Preclinical evidence	Clinical evidence	References
Acetylcholinesterase inhibitors: Physostigmine, Donepezil, Rivastigmine, Galantamine	Increases synaptic acetylcholine by inhibiting its breakdown in the synaptic cleft	Reduces TBI-induced neuronal death, BBB disruption, vasogenic brain edema Preserves neurons hippocampal region, neurologic and motor function	FDA-approved for treating cognitive disorders, including moderate and severe TBI	Cardenas et al. (1994), McLean et al. (1987), Ballesteros et al. (2008), Blount et al. (2002), Bourgeois et al. 2002, Foster and Spiegel (2008), Fujiki et al. (2006, 2008), Hayashida et al. (2007), Kaye et al. (2003), Khateb et al. (2005), Liepert (2008), Lombardi (2008), Masanic et al. (2001), Morey et al. (2003), Sugden et al. (2006), Taverni et al. (1998), Tenovuo (2005), Trovato et al. (2006), Walker et al. (2004), Wheaton et al. (2011), Whelan et al. (2000), Whitlock (1999), Zhang et al. (2004), Chen et al. (1998a, b), Noble and Hauser (2007), Silver et al. (2006, 2009), Tenovuo et al. (2009), Weinstock et al. (2001)
Huperzine A	A selective and reversible acetylcholinesterase inhibitors extracted from a Chinese herb An NMDA antagonist Neuroprotective effects through the activation of cholinergic systems and by potentially upregulating β-amyloid precursor protein metabolism	Modulates both amyloidogenic and non-myloidogenic pathways Attenuates cognitive deficits Improves memory	Huperzine A is classified as an herbal remedy, has beneficial effects in cerebral blood flow, general cognitive function, global clinical status, behavior disturbance, and functional performance.	Zhang et al. (2008), Wang et al. (2002, 2012), Fan et al. (2005), Li et al. (2008), Amen et al. (2011)

Lithium	Exerts neuroprotective effects through reduction of excitotoxicity, ischemic damage, and apoptosis Attenuates several pathways involving pro-inflammatory cytokines, β-APP-cleaving enzyme-1 expression, β-amyloid accumulation, microglial activation, cyclooxygenase-2 activity, glycogen synthase kinase-3β activity, and MMP-9 expression Preserves the integrity of the BBB	Attenuates Aβ load, amyloid precursor protein load, β-APP-cleaving enzyme-1 overexpression, Tau protein phosphorylation, TBI-induced neuronal death Reduces brain edema, neuronal and hemispheric volume loss, levels of IL-1β Improves spatial learning and memory	The primary drug for the treatment of bipolar disorder	Shapira et al. (2007), Yu et al. (2012a, b), Zhu et al. (2010), Dash et al. (2011), Bellus et al. (1996), Glenn et al. (1989), Haas and Cope (1985), Hale and Donaldson (1982), Parmelee and O'Shanick (1988), Schiff et al. (1982)
N-acetyl cysteine	Deacetylates to cysteine in the liver Both N-acetyl cysteine and cysteine are anti-oxidants that largely scavenge cytosolic radicals Increases levels of the endogenous anti-oxidant glutathione and extracelullar levels of glutamate	Anti-inflammatory activity by decreasing the activation of NF-kB, lowering IL-1β, TNF-α, and ICAM-1 levels Reduces lesion volume, levels of the putative neuroprotective enzyme heme oxidase Prevents myelin loss Restores memory	Significant increases in symptom resolution in individuals who suffer post-concussive symptoms	Abdel Baki et al. (2010), Atkuri et al. (2007), Dodd et al. (2008), Olive et al. (2012), Hicdonmez et al. (2006), Chen et al. (2008c), Yi and Hazell (2005), Hanci et al. (2010), Thomale et al. (2006), Hoffer et al. (2013)

(continued)

Table 2.1 (continued)

Compound	Neuroprotective mechanism	Preclinical evidence	Clinical evidence	References
Cyclosporine A	Inhibits opening of the mitochondrial permeability transition pore, and protein phosphatase calcineurin Reduces reactive oxygen species	Have beneficial effects on axonal injury, contusion volume, cerebral microcirculation	Phase II clinical trial, a positive effect on 6-month Glasgow Outcome Scale score was observed in TBI patients	Abdel Baki et al. (2010), Brustovetsky and Dubinsky (2000), Sharov et al. (2007), Alessandri et al. (2002), Büki et al. (1999), Colley et al. (2010), Kilbaugh et al. (2011), Mbye et al. (2009), Okonkwo and Povlishock (1999), Okonkwo et al. (2003), Scheff and Sullivan (1999), Setkowicz and Guzik (2007), Signoretti et al. (2004), Suehiro and Povlishock (2001), Sullivan et al. (1999, 2000a, b, 2011), Turkoglu et al. (2010), Van Den Heuvel et al. (2004), Gijtenbeek et al. (1999), Wijdicks et al. (1996), Empey et al. (2006), Mazzeo et al. (2006, 2008, 2009), Hatton et al. (2008)
FK 506	Inhibits calcineurin	Have beneficial effects on axonal injury, cerebral microcirculation	Chronic neurotoxicity	Singleton et al. (2001), Campbell et al. (2012), Oda et al. (2011), Piilgaard et al. (2011), Rhodes et al. (2009), Saganová et al. (2012), Grimbert et al. (1999)
Methylphenidate	Increases synaptic dopamine by blocking dopamine transporters and inhibiting dopamine reuptake Enhances synaptic norepinephrine levels by blocking norepinephrine reuptake	Promotes striatal dopaminergic neurotransmission Enhances spatial learning and retention and motor performance	FDA-approved for the treatment of attention deficit hyperactivity disorder Ongoing clinical trials in TBI patients	Moeller et al. (2012), Volkow et al. (1998, 2001, 2002a, b, 2012), Koda et al. (2010), Marsteller et al. (2002), Wagner et al. (2007, 2009a, b), Kline et al. (2000), Alban et al. (2004), Willmott and Ponsford (2009), Kim et al. 2006, 2012), Whyte et al. (1997, 2004), Plenger et al. (1996), Speech et al. (1993), Mahalick et al. (1998), Lee et al. (2005), Mooney and Haas (1993), Williams et al. (1998), Gualtieri and Evans (1988), Moein et al. (2006)
Atomoxetine	Inhibits norepinephrine transporters Increases extracellular norepinephrine and dopamine.	Enhances spatial learning and retention and motor performance	ibid	Koda et al. (2010), Swanson et al. (2006), Bymaster et al. (2002), Reid and Hamm (2008)

Erythropoietin	A pleiotropic cytokine involved in erythropoiesis Attenuates glutamate and nitric oxide toxicity, anti-apoptotic, anti-oxidant, and anti-inflammatory effects Stimulates neurogenesis and angiogenesis Protects mitochondria	Increases hematocrit by 60%	Ongoing Phase III clinical trials are safe and beneficial, resulting in lower hospital mortality	Jin et al. (2011), Valable et al. (2010), Xiong et al. (2008a, b, 2009, 2010a, b, 2011), Yatsiv et al. (2005), Zhang et al. (2009, 2010), Lu et al. (2005), Akdemir Ozisik et al. (2007), Bian et al. (2010), Chauhan and Gatto (2010), Chen et al. (2007b), Cherian et al. (2007), Cherian et al. (2011), Hartley et al. (2008), Liao et al. (2009), Lieutaud et al. (2008), Oztürk et al. (2008), Verdonck et al. (2007), Gonzalez et al. (2007), Ehrenreich et al. (2009), Tseng et al. (2009), Heeschen et al. (2003), Miskowiak et al. (2010), Wüstenberg et al. (2011)
Glyburide	A sulfonylurea binds sulfonylurea receptors blocking ATP-sensitive K^+ channels in neurons.	Reduces inflammation, hemorrhage, vasogenic edema, hippocampal injury	Ongoing Phase II clinical trial shows changes in edema and/or hemorrhage in moderate or severe TBI	Silver et al. (2009), Simard et al. (2009, 2012), Patel et al. (2010), Ortega et al. (2012), Kunte et al. (2007)
Minocycline	Has anti-inflammatory, anti-apoptotic, and anti-oxidant activity	Inhibits microglia, T cells, neutrophils, metaloproteases, apoptosis, reactive oxygen species Reduces lesion volume Maintains myelin content Protects grey and white matter	Confirmed as an anti-inflammatory drug	Abdel Baki et al. (2010), Kim and Suh (2009), Bye et al. (2007), Homsi et al. (2010), Sanchez Mejia et al. (2001), Kovesdi et al. (2012), Casha et al. (2012), Racette (2008), Sacktor et al. (2011)
Statins: Simvastatin	Has beneficial effects in brain edema, BBB integrity, cerebral blood flow, neuroinflammation, axonal injury, cell death, trophic factor production	Against neuronal death	Ongoing Phase II clinical trial in adults with TBI	Chauhan and Gatto (2010), Abrahamson et al. (2009), Béziaud et al. (2011), Chen et al. (2008d, 2009), Indraswari et al. 2012, Li et al. (2009), Lu et al. (2004a, b, c, 2007), Wang et al. (2007), Sierra et al. (2011), Boimel et al. (2009)

(continued)

Table 2.1 (continued)

Compound	Neuroprotective mechanism	Preclinical evidence	Clinical evidence	References
Amantadine	Increases extracellular dopamine by blocking dopamine reuptake, facilitating dopamine synthesis Has postsynaptic effects on dopamine circuits by increasing dopamine receptor density antagonist of NMDA receptors		Phase III clinical trial in 184 patients in the vegetative state or minimally conscious state shows improvement in the Disability Rating Scale, representing the first and only evidence to date that pharmacologic intervention can affect recovery from TBI.	Bales et al. (2009), Gianutsos et al. (1985), Stoof et al. (1992), Dixon et al. (1999), Meythaler et al. (2002), Schneider et al. (1999), Whyte et al. (2005), Giacino et al. (2012)

Abbreviations: *IL* interleukin, *TNF* tumor necrosis factor, *NFkB* nuclear factor kappa-light-chain-enhancer of activated B cells, *TBI* traumatic brain injury, *FDA* the U.S. Food and Drug Administration, *BBB* blood brain barrier, *NMDA* N-methyl-D-aspartate, *Aβ* β-amyloid, *MMP* matrix metalloproteinase, *ICAM* intercellular adhesion molecule

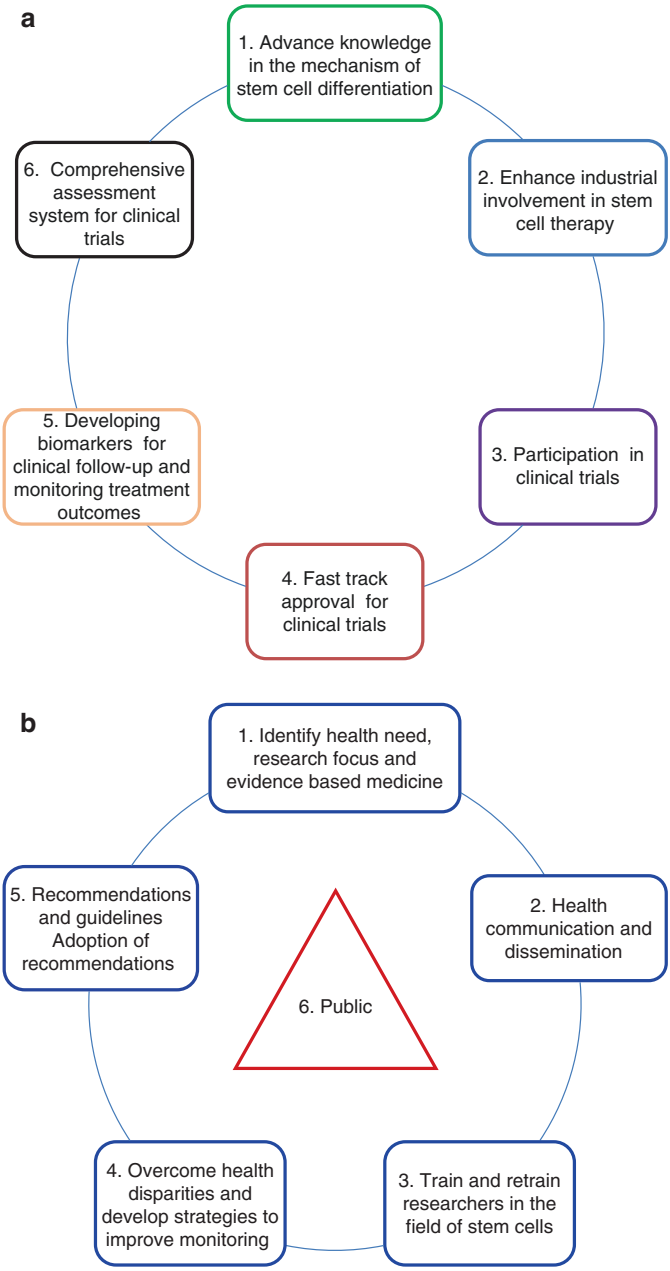

Fig. 2.3 (**a**) Proposed schema for effective translation of stem cells to therapy in traumatic brain injury involving concerted effort of multilevel strategies of six main stakeholders. (**b**) Proposed framework for the reinforcement of the multilevel strategies effective bench to bedside translation of stem cells to therapy in traumatic brain injury

(Nakamura et al. 2003). The efficacy of transplantation largely depends on a grafting method that optimizes the survival of the transplanted stem cells and minimizes the graft-induced lesion. Most transplantation studies involved intraparenchymal injection into the central nervous system, in which cells were grafted directly into or adjacent to the lesion (Chow et al. 2000; Cao et al. 2001; Jendelová et al. 2004). The optimal time for transplantation may not be immediately after injury. The levels of various inflammatory cytokines (tumor necrosis factor alpha, interleukin-1α, interleukin-1β and interleukin-6) in the injured brain peak 6–12 h after injury and remain elevated until the 4th day. Although these inflammatory cytokines are known to have both neurotoxic and neurotrophic effects, they are believed to be neurotoxic within a week after injury, which causes the microenvironment to be unsuitable for survival of the grafted stem cells (Zhu et al. 2006). However, if too much time passes after the injury, glial scar forms a barrier surrounding the lesion site and inhibits revascularization of the graft preventing local blood circulation which is needed for graft survival. Thus, it is considered those 7–14 days after traumatic brain injury is the optimal time for stem cell transplantation (Ogawa et al. 2002; Okano et al. 2003).

2.4.2 Stimulation of Endogenous Neural Precursor Cells in Traumatic Brain Injury

Endogenous neurogenesis has been identified in adult brain (Luskin et al. 1996; Alvarez-Buylla et al. 2000). In adult rodent brain, neural stem cells migrate from ventricular zone to the olfactory bulb and integrate into the neuronal network. This is called the rostral migratory stream. However, the potential success of stimulating endogenous neural precursor cells is hinged on delivery of various growth factors. This is the most common way to stimulate neural precursor cells. The following growth factors are needed to stimulate neural precursor cells: epidermal growth factor, fibroblast growth factor-2 (Martens et al. 2002; Kojima and Tator 2000, 2002), basic fibroblast growth factor (Rabchevsky et al. 2000), acidic fibroblast growth factor (Lee et al. 2004), brain-derived neurotrophic factor (Namiki et al. 2000; Wang et al. 2013), vascular endothelial growth factor (Sharma 2003; Chang et al. 2013), nerve growth factor, neurotrophin-3 (Namiki et al. 2000; Widenfalk et al. 2003), glial cell line-derived neurotrophic factor (Iannotti et al. 2004), insulin-like growth factor-1 (Sharma 2003), and stromal cell-derived factor-1 alpha (Imitola et al. 2004). They were administrated by intraventricular (Martens et al. 2002), intraparenchymal (Namiki et al. 2000; Sharma 2003) or intrathecal (Kojima and Tator 2000, 2002; Rabchevsky et al. 2000; Iannotti et al. 2004) injection. They were reported not only to enhance the proliferation, migration and gliogenesis of neural precursor cells (Martens et al. 2002; Kojima and Tator 2000, 2002; Imitola et al. 2004) but also to protect the spinal cord from further damage (Sharma 2003; Widenfalk et al. 2003). In addition, these growth factors facilitate the regrowth of

axons and remyelination (Lee et al. 2004; Namiki et al. 2000; Gensert and Goldman 1997). Functional recovery has been reported after growth factors were delivered into injured spinal cord (Martens et al. 2002; Kojima and Tator 2000, 2002; Lee et al. 2004). However, the mechanisms of functionary recovery by stimulating endogenous neural precursor cells are not fully understood.

In addition to growth factors, other molecules are shown to stimulate endogenous neural precursor cells. Proliferation of endogenous neural precursor cells was demonstrated when the sodium channel blocker, tetrodotoxin and the glycoprotein molecule, sonic hedgehog were injected into the parenchyma (Rosenberg et al. 2005; Bambakidis et al. 2003). It has been reported that cognate chemokine receptor type 4 expressed by neural precursor cells can regulate their proliferation and direct their migration towards the injury site (Imitola et al. 2004). In addition, antibodies blocking interleukin-6 receptors were reported to not only inhibit differentiation of endogenous neural stem cells into astroglia *in vivo* and *in vitro*, but also to promote functionary recovery (Okada et al. 2004; Nakamura et al. 2005). The functionary recovery is resulting from blocking interleukin-6 and consequently inhibiting the formation of glial scars and promoting axonal regeneration (Okada et al. 2004; Okano et al. 2005). Notably, studies of ATP-binding cassette (ABC) transporters have emerged as a new field of investigation. ATP-binding cassette transporters, especially ATP-binding cassette sub-family A member 2, ATP-binding cassette sub-family A member 3, ATP-binding cassette sub-family B member 1, and ATP-binding cassette sub-family G member 2, play an important role in proliferation and differentiation of neural stem cells (Lin et al. 2006; Eckford and Sharom 2006; Leite et al. 2007; Li et al. 2007; Saito et al. 2007; Tamura et al. 2006).

In contrast to transplantation of exogenous neural precursor cells, stimulation of endogenous neural precursor cells to repair damaged spinal cord has three main advantages: (1) there is no ethical issue involved in human embryonic stem cells, (2) it is usually less invasive since no surgical procedure required, and (3) no immunogenicity, which avoids immunorejection that observed in the transplantation of exogenous neural precursor cells (Mohapel and Brundin 2004). Similar to the transplantation studies of adult neural precursor cells in spinal cord injury, no neurogenesis has been reported from the stimulation of endogenous neural precursor cells. It has been reported that up-regulation of the Notch signal pathways leads to poor neuronal differentiation (Yamamoto et al. 2001). The increased levels of various cytokines within the microenviroment surrounding the area of injury cause a lack of trophic support for differentiation of neural precursor cells into neuronal lineage (Okano et al. 2003; Frisén et al. 1995; Johansson et al. 1999; Widenfalk et al. 2001).

Recently, more attention has been drawn to cAMP response element binding protein/p300-phosphorylated Smad protein complex. It was found that cAMP response element binding protein/p300-phosphorylated Smad protein complex can be bound in neural stem cells, which determines the differentiation of neural stem cells. If the complex is bound with phosphorylated signal transducers and activator of transcription 3, the neural stem cells differentiate into astroglia lineage cells. On the other hand, if the complex is bound with proneural-type of the basic

helix-loop-helix factor, such as neurogenin 1 and 2, they differentiate into the neuronal lineage (Okano et al. 2005; Sun et al. 2001; Nakashima et al. 1999). Apart from that, SOX gene may also play an important role in neural differentiation (Pevny and Placzek 2005). Once neural stem cells decide to differentiate into neuronal lineage, a cascade of hundreds of genes is regulated over time to lead the immature neuron into its mature phenotype. Many of these neural genes are controlled by RE1-silencing transcription factor. RE1-silencing transcription factor acts as a repressor of neural genes in non-neural cells, while regulation of RE1-silencing transcription factor activates large networks of genes required for neural differentiation (Gage and McAllister 2005; Ballas et al. 2005; Ballas and Mandel 2005).

2.5 Bench to Bedside Translation of Stem Cell Therapy in Traumatic Brain Injury

The main purpose of state-of-the-scientific studies is to translate our discoveries into daily clinical practice. The basic research laboratory takes its observations obtained at molecular or cellular levels in a cutting edge state and implements this into acceptable clinical practice to the benefit of the public. However, this is always met with a lot of challenges, such as ethics, governmental regulations, funding constraints, paucity of adequate collaboration among clinical and basic scientists, and the challenges during conducting clinical trials. From the identified gaps in the current state of the stem cell science and inherent challenges faced by the field, the author proposes six point schema for improving bench to bedside translation of stem cell therapy in Fig. 2.3a involving a rigorous network of six stakeholders: basic researchers, pharmaceutical companies, patients or general public participating in clinical trials, regulatory bodies or government agencies for providing research grant approval, collaborative research between basic and clinical scientists with the plan of developing biomarkers for potential drug targets and creating a concerted network of groups that identifies some of the medical problems relating to traumatic brain injury. Patients with moderate traumatic brain injury who suffer long-term complications are a major unmet medical need. Within our capabilities to clinically assess improvement, historically, the majority of individuals with moderate traumatic brain injury are likely to recover to their pre-injury state. Early identification of those individuals likely to experience long-term complications is essential to maximize benefit of stem cell therapy. Strategies to delineate this population from a larger population of individuals with moderate traumatic brain injury could include enrollment of patients with persistent symptoms 1–2 weeks after injury, because recovery is most rapid in the first few days. Patients who are unlikely to fully recover could be identified using prognostic biomarkers including neuroimaging, biochemical, and objective clinical measures. Prognostic biomarkers are defined by the U.S. Food and Drug Administration as indicators that inform the natural history of a disorder in the absence of a therapeutic intervention (Drug Administration 2010).

Although identifying individuals with traumatic brain injury who are most likely to respond to stem cell therapy and evaluating the biologic response to the therapy are essential for successful clinical trials, the ability to do either is lacking. Predictive biomarkers of stem cell therapeutic response are needed to address this challenge. Predictive biomarkers are baseline characteristics that identify individuals by their likelihood to respond to a stem cell therapy and may include biochemical markers including oxidative stress, inflammation, neuronal, and glial integrity, molecular imaging with positron emission tomography, or functional imaging with functional magnetic resonance imaging. By identifying patients who are most likely to respond to stem cell therapy, the appropriate population can be selected for enrollment in clinical trials. Identifying specific predictive biomarkers would decrease the sample size needed to power clinical trials, thus decreasing risk to subjects, time to complete accrual, and cost. Biomarkers are dynamic measurements that show a biologic response occurred after stem cell therapy, including neuroimaging to measure effects on neuroprotection, neurorecovery, and neuroinflammation, or biochemical biomarkers of oxidative stress, inflammation, and neuronal integrity. Clinical trials would greatly benefit from biomarkers, which allow for the measurement of the effect of the stem cell therapy on the putative mechanism of a specific phenotype of cell's action, thus providing evidence of engagement of the target tissue by the therapy. To achieve stem cell repair, regeneration and protection after traumatic brain injury, each of the six points identified is critical for advancing the field, and efforts to address the points should be conducted in parallel to ensure ultimate success in improving clinical care and outcomes for individuals with traumatic brain injury. We are still faced with the need to formulate hypothesis both at experimental and clinical epidemiologic level and implementing these into clinical practice while the translational researchers serve to collaborate and coordinate all these strategies to yield rapid results.

Indeed, communication and dissemination shown in Fig. 2.3b which is patient centredness will not only impact on the public, but will also help to tame the ethical issues in this field. Communication will involve both patients and clinicians involve in conducting randomized clinical trials. With strong feedback on outcomes, pharmacovigilance, and health promotion. Education of the populace in form of scientific advocacy is so paramount as this will impact on improved scientific collaboration, quality public control, and increased transparency among researchers and may improve funding of research work (Keramaris et al. 2008).

Research in neural stem cells is still a grey area and much knowledge needs to be gained at the bench in order to actually close the knowledge gaps in stem cell therapy. There is inadequate understanding of the secondary brain injury process after traumatic brain injury, insufficient preclinical testing in diffuse axonal injury models, species differences, and lack of understanding of the mechanism of drug-receptor interactions. It has been suggested the need to use gyrencephalic models for proper translation of stem cell therapy in traumatic brain injury (Loane and Faden 2010). Academic and biotech researchers should address how to make their stem cell therapy products more feasible for commercial-scale production (Eaker

et al. 2013). There is need for increased linkages and networking between academician, researchers, and clinician for greater reward of what is being generated.

Methodological disparities between experimental models of traumatic brain injury and clinical studies cannot be overemphasized. The intent to treat models, differences in statistical analysis as a result of different sample size, and different behaviours between human and animals. Animal research is a rapid, well-controlled, and cost-effective means to initially verify hypothesis. However, limitations exist in animal models of traumatic brain injury and their application in stem cell therapy. First, because no single animal model accurately mimics all of the features of human traumatic brain injury, individual investigators have appropriately refined experimental approaches to better fit their specific research goals. However, the resulting variability in experimental approaches among studies makes comparison of results across laboratories and models difficult, limiting the confidence that results can be translated into successful clinical trials. Advancing preclinical research in animal models requires that results are comparable across studies and can translate into human studies. This requires standardization of available animal models and introduction of new models when scientifically necessary. Second, some of the popular current models do not correspond well with the human condition. Injury severities in animals differ from humans; while they are well defined in animals, it could take any direction in human. Third, preclinical studies should use the same level of rigor required for clinical trials. Specifically, assignment of animals to treatment conditions should be randomized, assessments must be conducted by blinded examiners, the primary outcome measure must be pre-determined, and statistical assessment of secondary outcome measures should utilize appropriate corrections for multiple comparisons. Fourth, the transplantation of stem cells into animal models should mimic the timing, delivery route, and equivalent mass of cells feasible in humans. Last, the neurobehavioral outcome measures most widely used in preclinical models are not sufficiently sensitive to long-term behavioral and cognitive deficits, and more sensitive rodent behavioral tasks that discriminate injury severity beyond 12 weeks after injury are needed. The need to improve study quality score has recently being called for by stroke therapy academic industry roundtable, which was recently updated and this include the following recommendations: (1) Elimination of randomizations and assessment bias, (2) Use of a priori definitions of inclusion/exclusion criteria, (3) inclusion of appropriate power and sample size calculations, (4) full disclosure of potential conflict of interests, (5) evaluation of therapies in male and female animals across the spectrum of ages, and with comorbid conditions, such as hypertension and/or diabetes. Furthermore, some researchers has expanded on these proposed recommendations for improved clinical trials in brain injury with special focus on neuroprotective therapies in traumatic brain injury (Loane and Faden 2010; Fisher et al. 2009). Nonadherence was the single most important determinant of trial failure in the past.

Finally, the International Mission on Prognosis and Clinical Trial Design in traumatic brain injury proposed ways of overcoming the above disparities and challenges. The recommendations include a robust inclusion criteria and recommendations for general research in traumatic brain injury (Loane and Faden

2010). The six point schema is an overview recommendation with the public, patient or the society as the core and the fulcrum of all activities of research and if implemented may yield quality research outcome in neural stem cells therapy in traumatic brain injury (Ugoya and Tu 2012).

2.6 Conclusion

Mortality and long-term disability from traumatic brain injury is projected to rise globally. Neural stem cell therapy is a strategy that offers hope for the future in treatment of brain injury. In addition, we are now able to monitor autologous neural stem cells *in vivo*, cell migration, and clearly demonstrate that neural stem cells could selectively target injured brain or spinal cord tissue and undergo neurogenesis. Finally, the proposed six points cyclical schema should be implemented with determined effort of all stakeholders for effective bench to bedside translation of neural stem cell therapy in traumatic brain injury.

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Chapter 3
Stem Cells in Alzheimer's Disease Therapy

Atipat Patharagulpong

3.1 Introduction

Alzheimer's disease (AD), the most prevalent form of dementia (60–70 % of cases) (Burns and Iliffe 2009; World Health Organization 2012), is a terminal neurodegenerative disease, leading to a life expectancy of 3–9 years after diagnosis (Todd et al. 2013). As neural loss occurs over time, the early symptoms begin with short-term memory loss, clumsiness, and depletion of language competency. The disease then progresses to long-term memory impairment, mood swings, disorientation, and loss of motivation, with the final stage leaving the patient with emotional apathy, loss of speech (Frank 1994), extreme exhaustion, and an inability to perform daily tasks as a result of mobility deterioration (Förstl and Kurz 1999). The current number of AD and related dementia cases is estimated to be 46.8 million worldwide in 2015 while the cost is estimated $818 billion, 1.09 % of the global domestic product (Prince et al. 2015). AD is therefore a major burden to patients and society (Meek et al. 1998), and the therapeutic strategy to tackle the disease is required as soon as possible.

There are major challenges in defeating AD. First of all, the disease itself is difficult to diagnose in its early stages as it requires at least 8 years for a patient to develop full AD symptoms. The patient is normally given a behavioral and cognitive test, as well as a physical examination, to identify the disease (Scott and Barrett 2007). However, these are still not 100 % accurate as the disease shares traits with other cerebral pathologies (The dementias: hope through research 2013). The use of medical imaging could exclude other symptom-related diseases (Weiner 2009), but the cost and worldwide accessibility of the procedure are still problematic, leading to additional delay to the disease detection. Furthermore, the multifactorial and

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complex nature of AD creates difficulties in therapeutic development (Carreiras et al. 2013), as one particular drug could not entirely cure the whole range of symptoms posed by the disease. The current drugs also cause various side effects in the long term and often fail to cure late stages of the disease as a result of excess cortical neural loss (Golde et al. 2011). Stem cell therapy which can be used to differentiate stem cells into different neuronal types and replenish significant neural loss in the later stage of the disease is therefore a major candidate for the therapeutic strategy of AD.

AD could be subdivided into two categories. The early-onset or familial type (fewer than 5 % of the cases) is found in people younger than 65 years old, whereas the 65 and older demographic predominates for the late-onset or non-familial case (Blennow et al. 2006). Even though the symptoms of both diseases subtype are similar, the molecular pathophysiology is quite different. The responsible pathogenic molecules and the drugs targeting them, in addition to the reason for stem cell therapy being more desirable than the current pharmaceutical approach, will be discussed in the next section.

3.2 Pathogenesis of Alzheimer's Disease and Drug Treatment

In general, patients with AD show significant neuronal and synaptic loss in different regions of the brain, including parietal and temporal lobes as well as the cingulate gyrus and parts of the frontal cortex (Wenk 2003). The level of neurotransmitters in the AD brain has significantly altered. This includes an increase in an excitatory neurotransmitter glutamate (Dong et al. 2009), which causes excitotoxicity and a decrease in an inhibitory neurotransmitter acetylcholine (Baskys 2006). Acetylcholine functions to modulate neuronal plasticity which is crucial in the building up and maintenance of memory, as well as arousal, reward and sustaining attention (Jones 2005). Therefore, its falling level in AD leads to memory loss and depression. The deficiency in acetylcholine production was once believed to be the main cause of AD development, however, this hypothesis was proven to be wrong as drugs, (e.g. donepezil), that inhibit acetylcholinesterase, the enzyme that breaks down acetylcholine, could only treat the symptoms but never halt the progression of the disease (Shen 2004). It is therefore concluded that the reduction in acetylcholine level is not the main cause but rather the result of the loss of neural tissue producing it (Wenk 2003).

With further studies, it has been identified that AD is mainly caused by protein misfolding. The first misfolded protein that is a hallmark of AD is beta-amyloid (Aβ), which is a peptide of 39–43 amino acids. In familial AD, 42 amino-acid Aβ (10 % of Aβ released from cells) (Maslow 2008) are mainly responsible for the early onset of the disease and are a major component of neuritic plaques (Fig. 3.1), while diffusible and less hydrophobic 40 amino-acid Aβ (90 % of Aβ released from cells) contribute only to later stages of AD pathology and are found mainly in cerebrovascular plaque (Lue et al. 1999). These plaques are composed of insoluble amyloid

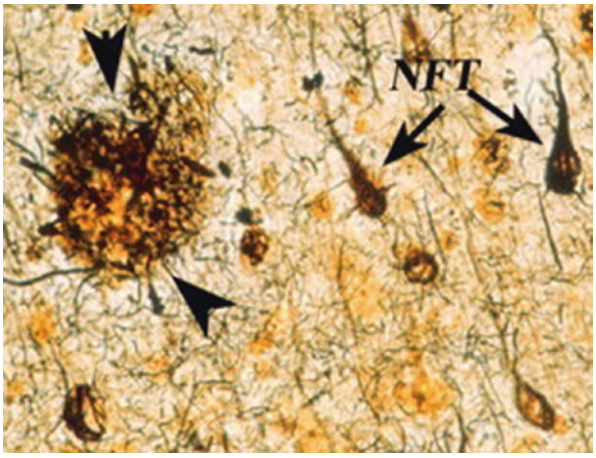

Fig. 3.1 Aβ plaques (*arrowheads*) and neurofibrillary Tau tangles (*arrow*) in AD brain stained with Bielschowsky silver stain (Nixon 2007)

fibres, which are regularly arranged aggregates. The neuritic Aβ plaques are deposited in extracellular site of cortical and hippocampal neurons of the AD brain (Sadigh-Eteghad et al. 2014). In the Aβ hypothesis, the Aβ plaque is believed to be the origin of the AD hallmark, which causes cytotoxicity by clogging up intercellular communication, activating oxidative damage and immune response that elicits inflammation and microglial activation, ultimately proceeds to cellular apoptosis (Glass et al. 2010) (Fig. 3.2). The higher production of $A\beta_{42}$ in the familial AD results from mutations of the proteins in Aβ production pathway (Fig. 3.3). Amyloid precursor protein (APP), which anchors in the neuronal membrane, is cleaved by β-secretase to release soluble Aβ and γ-secretase to produce 42 or 40 amino acids. The mutation could occur at the secretase cleavage sites of APP, which causes the secretase to produce higher proportion of the longer $A\beta_{42}$ (Mullan et al. 1992). Another mutation could also occur at the catalytic subunit of the γ-secretase protein complex, which is encoded by presenilin1 (PS1) or presenilin2 (PS2), and result in the aggregated Aβ plaque production (Huang and Mucke 2012). The duplication of the APP gene by itself or by trisomy of chromosome 21 also elevates the amount of Aβ plaque in the brain (Goate et al. 1991; Prasher et al. 1998). Aβ plaque was found to involve in neurogenesis, the process where new neurons are generated from neural stem cells or neural precursor cells and migrates to the olfactory bulb or the hippocampus. This is important as immature neurons that migrate to the hippocampus could mature into memory-related cholinergic neurons, the main population that is destroyed in AD. It is found that the proliferation and migration of neuronal progenitor cells were impaired in APP mutant mice or mice infused with Aβ (Haughey et al. 2002), therefore, familial AD could potentially result in reduction of neurogenesis.

In non-familial AD cases, which do not involve the APP processing gene mutation, apolipoprotein (Apo)E4 is validated as the main genetic risk (Corder et al.

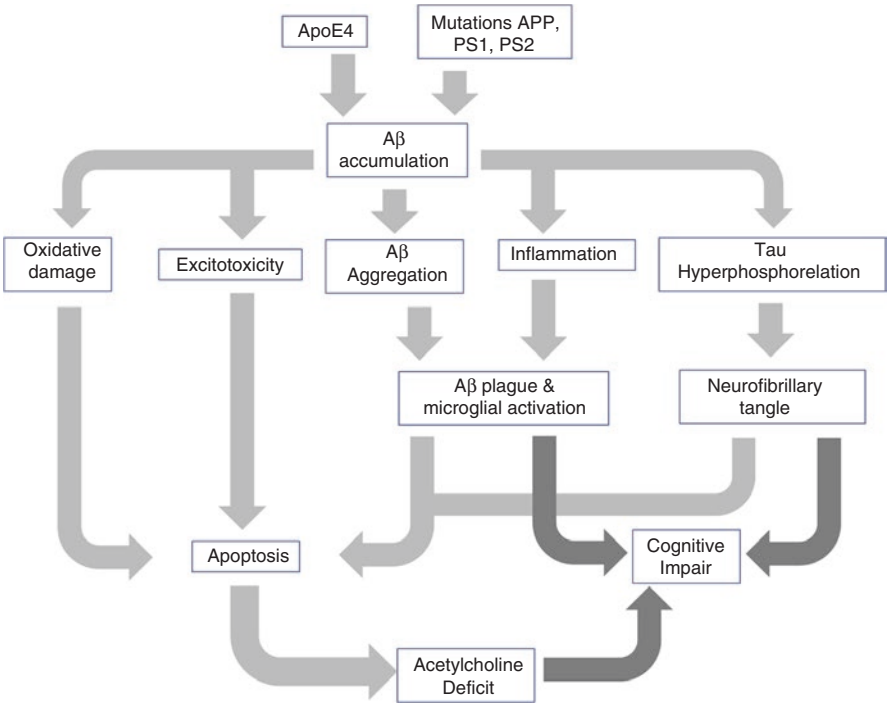

Fig. 3.2 Diagram showing the sequential pathological effects of non-familial (ApoE4 expression) and familial (APP, PS1, and PS2 Mutations) AD (Blennow et al. 2010) (*ApoE4* Apolipoprotein4, *APP* Amyloid precursor protein, *PS* presenilin, *Aβ* beta amyloid)

2013). ApoE is a protein involved with triglyceride-rich-lipoprotein catabolism which is encoded by three alleles into three isoforms: ApoE2, ApoE3, and ApoE4. ApoE, in general, catalyzes the breakdown of the fibrillary Aβ plaque. ApoE3 is the neutral form which is found mainly in non-patients, while the rarest form, ApoE2, with higher catalytic efficiency than ApoE3, could reduce the risk of AD (Farrer et al. 1997). However in non-familial AD cases, allele ApoE4 is mostly present where the ApoE4 protein shows lower catalytic efficiency and poses a 20 times higher risk of AD (Jiang et al. 2008; Hauser and Ryan 2013).

There have been different attempts to cure AD by targeting Aβ production, aggregation, and removal. To reduce Aβ production, a drug containing γ-secretase inhibitor could be employed to halt the APP clipping, however it is shown to cause abnormalities in the gastrointestinal tract, spleen, and thymus in the rat model, as a resulting inhibition of Notch (multi-function signaling molecule) cleavage (Imbimbo and Giardina 2011). Furthermore, a drug such as R-flurbiprofen was also designed to reduce $A\beta_{42}$ production by modulating the APP cutting of secretase into different fragments rather than $A\beta_{42}$, however, this failed in Phase III trials. Another Phase III drug, Tramiprosate, was developed to block Aβ aggregation, but was halted as a result of high data variation among trials (Karran and Hardy 2014). Many vaccines composed of Aβ fragments to trigger

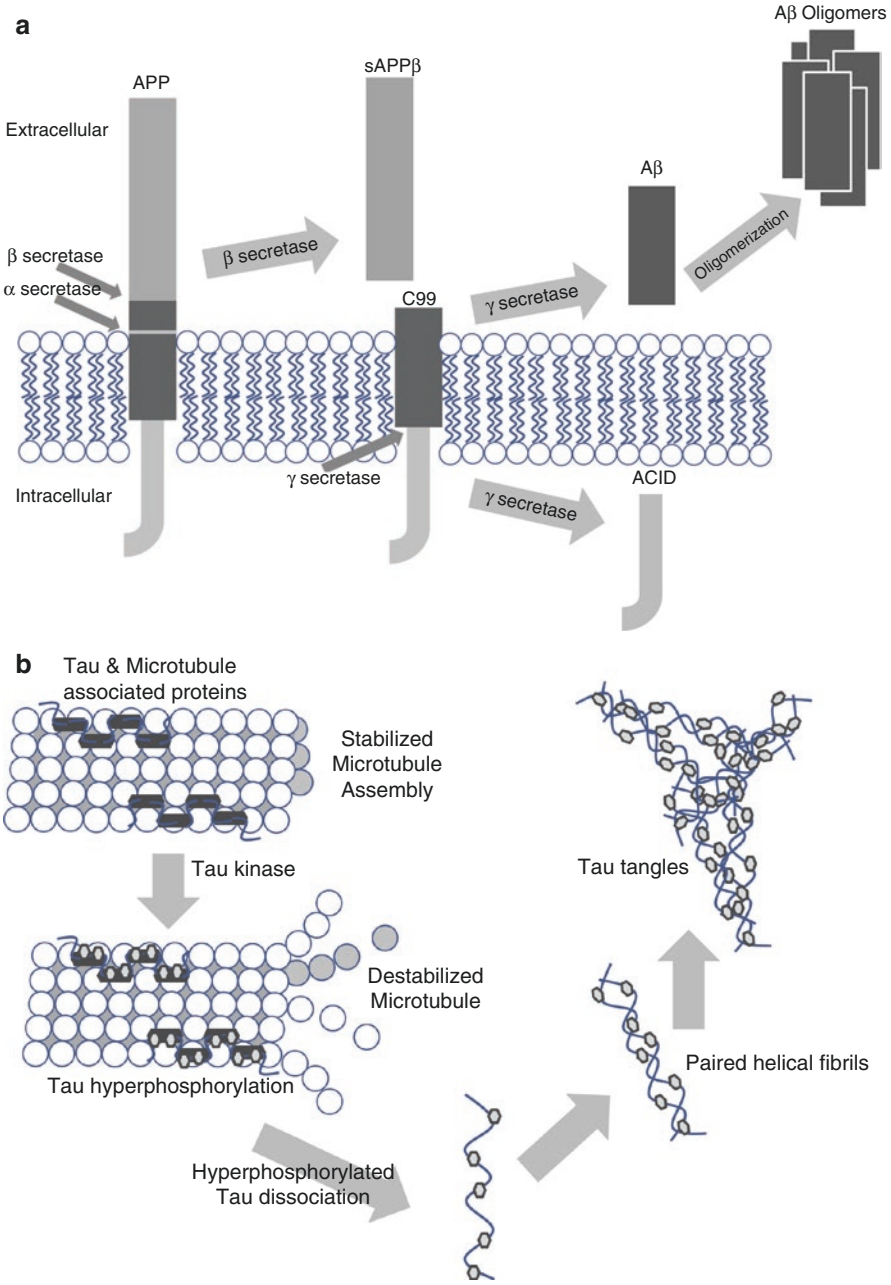

Fig. 3.3 (**a**) APP could undergo proteolytic cleavage and result in the production of Aβ, which aggregates to form Oligomeric plaques (Nicolas and Hassan 2014). (**b**) Hyperphosphorelation of Tau causes the protein to dissociate from microtubules, results in microtubule's destabilization. Tau then aggregates to form neurofibrillary tangles (Simic et al. 2016) (*APP* amyloid precursor protein, *sAPPβ* soluble ectodomain APPβ, *C99* C-terminal fragment 99, *Aβ* beta amyloid, *ACID* APP intracellular domain)

immune response, as well as natural antibodies, were developed to remove Aβ plaques from the brain (McLaurin et al. 2002). However, the treatment alone could not cure AD effectively in the later stage. For all these reasons, developing an effective Aβ medication with minimal side effects is still an outstanding challenge.

The second misfolded protein that is a hallmark of the AD is diffusible microtubule-associated protein Tau (Shin et al. 1991). Tau modulates the stability of the axonal microtubule by interacting with the tubulin protein, which is crucial for axon formation as well as maintaining cell structure and intracellular trafficking. In the Tau hypothesis, Tau is considered to be the primary cause of AD (Billingsley and Kincaid 1997). Tau in the AD brain is hyperphospohorylated: it aggregates to form oligomers and assembles into insoluble filaments and later tangles (Figs. 3.1 and 3.3). The Tau tangle not only reduces affinity to tubulin binding but also sequesters the normal Tau, MAP1, and MAP2 from binding to tubulin itself. Sequestration of these proteins inhibits microtubule assembly and promotes its disassembly (Alonso et al. 1997). As a result, the microtubule transport is impaired. The synaptic transmission, which requires the trafficking of synaptic vesicles and ion channels to the distal part of the neuron, is consequently blocked and the synapses degenerate, resulting in cognitive impair (Thies and Mandelkow 2007) (Fig. 3.2). The hyperphosphorylation of Tau is believed to result from the upregulation of its kinase such as cdk5 and the downregulation of its phosphatase such as PP2A in the AD brain. It is also hypothesized that Aβ could promote Tau hyperphosphorylation by upregulating a Tau kinase GSK3β, however study suggested impaired APP proteolytic processing, rather than Aβ accumulation, could promote this (Liu et al. 2006; Chabrier et al. 2012). Tau was also found to affect adult hippocampal neurogenesis by reducing the proliferation of stem/progenitor cells in dentate gyrus (Pristerà et al. 2013).

Currently, different Tau medications are still far from being complete, and are even less developed than Aβ medications. Most of the drugs target the hyperphosphorylation of Tau and its assembly (Brunden et al. 2009). However, the full treatment of AD by targeting Aβ or Tau alone is still underwhelming. Stem cells have come to light as an alternative therapy of AD, as the differentiated neuronal cells could replace the AD affected neurons and potentially recover the patient's brain to a normal state. The use of stem cells for AD therapy and the models used for its study will be discussed in the further sections.

3.3 Animal Models in AD Therapy with Stem Cells

Even though the stem cell treatment for AD has not been initiated in human, experiments in several animal models have validated its potential (Han et al. 2015). These models are mainly mice and rats since they are mammals and exhibit higher order brain functions like human. They have short lifespans of 1–3 years, allowing faster study of the disease progression, and their AD brains exhibit Aβ hallmark similar to humans (Webster et al. 2014). Different types of rodent models were studied for stem cell transplantation, including natural age-induced rats, Aβ-infused rats,

chemically-induced rats, surgery-induced rats, and transgenic mice (Wang et al. 2015). An Aβ-infused rat is achieved by intra-ventricular injection of Aβ plaques into its cortex or hippocampus. Despite not showing similar pathological progression of the disease, the downstream AD effects involving inflammation, neuro- and synaptodegeneration as well as motor and memory dysfunction are still present (Yun et al. 2013). Other chemicals induced for hippocampal lesions were mainly neurotoxins such as kainic acid (Park et al. 2012a) and ibotenic acid (Lee et al. 2012), which are glutamate agonists and mediate excitotoxicity, or scopolamine (Safar et al. 2016), which also causes similar cholinergic neural loss. Surgery induction could be achieved by olfactory bulbectomy (Bobkova et al. 2013) which demonstrate the main symptoms of AD type neurodegeneration, or fimbria fornix transaction (Gu et al. 2008), which shows similar neural loss effect in the basal forebrain of senile dementia patients. It is noticed that rats are preferred over mice in these models since they are physiologically, genetically, and behaviorally closer to human.

However, in transgenic models, mice are preferred over rats since the transgenic injection into more visible pronuclei through the more flexible pronuclear membrane could be performed more easily (Charreau et al. 1996). The transgenic approach was carried out by injecting a transgene composed of a coding region coupled to a promotor that drives expression into the pronuclei of one-cell staged embryo. Different gene modifications involved in Aβ or Tau pathways have been employed to create murine AD models. These include mutations of APP and PS1 genes. Originally APP (Tg2576) with APP overexpression or PS1 (M146L mutation) with PS1 overexpression alone was mutated in AD models, which showed similar Aβ deposition in AD. However the APP mutation did not exhibit taupathy (Goedert et al. 2006; Hsiao et al. 1996) despite the development of Aβ plaque and neurodegeneration (Kalback et al. 2002), while the PS1 mutation did not show abnormal pathological AD plaques deposition despite having high levels of $A\beta_{42}$ (Chui et al. 1999). This suggests a single gene mutation could not elicit complete AD symptoms. Later on, the triple mutation model of APP, PS1, and Tau was developed, allowing restoration of the taupathy showing Aβ pathology similar to AD in the long term (Oddo et al. 2003). Even though the triple mutation model could be used to study the disease's progression and inflammation as well as mitochondrial oxidative stress and dysfunction, was the progressive neurodegeneration in the hippocampus and other neocortical areas is not prominent.

3.4 Stem Cells as a Model to Study AD

Although rodent AD models have been developed to exhibit AD pathologies, the genetic discrepancies between rodents and humans still cause difficulties in terms of accurately modelling AD for full clinical implications. As an alternative, human stem cells could be differentiated into various cell types and used to examine the effect of AD molecular pathology. Examples of human stem cell sources include induced pluripotent stem cells (iPSCs) (Grskovic et al. 2011), which are generated

by reprogramming somatic cells such as blood, umbilical cord, skin, or fibroblast cells with four transcription factors: OCT4, SOX2, c-MYC, and KLF4. iPSCs were generated from AD patients with PS1 and PS2 mutations, and then further differentiated into neurons. The neurons were found to produce more $A\beta_{42}$ depositions: moreover, γ-secretase inhibitors and modulators could regulate plaque production (Israel et al. 2012). iPSC-derived neurons could therefore be a platform for development of drugs against AD.

In order to evaluate the effectiveness of stem cell transplants, the stem cell model was also exposed to AD pathological molecules or genetically engineered to express ones. For example, human embryonic stem cells (hESCs), the pluripotent stem cells derived from the blastocyst stage of the embryo, engineered as an AD model to overexpress wild-type or mutant-human APP, were found to differentiate spontaneously into neural lineage, despite being cultured in standard hESC media. The N-terminal fragment of APP resulted from proteolysis was found to be critical to this effect, which could support the importance of AD-related molecules in stem cell differentiation and neurogenesis (Freude et al. 2011). In another example, Neural precursor cells (NPCs), partially differentiated neuronal cells, were treated with Aβ40 or $A\beta_{42}$ for 24 h; the former treatment, was found to increase the number of neurons significantly, whereas the latter increased astrocytes instead (Chen and Dong 2009). The forms of Aβ also affect neurogenesis in AD. It was found that neural precursor cells proliferate significantly with soluble $A\beta_{42}$ while fibrillar $A\beta_{42}$ reduces or does not affect neurogenesis (Heo et al. 2007). Therefore, eliminating fibrillar $A\beta_{42}$ might be the key to yield a more effective stem cell therapy.

3.5 The Use of Stem Cells in AD Therapy

Since significant numbers of AD drug development efforts have failed in late-phase clinical trials, other approaches including cell-based therapies have emerged as alternatives. The problems with conventional cell therapy occur because different neuronal systems with multiple neurotransmitter phenotypes are stochastically affected in AD, leading to difficulty in accurately targeting all affected areas. One way to potentially overcome this is to use neural stem cells (NSCs), the cells that could give rise to different cell types in neural lineage, which could migrate from injection sites to different areas in the brain, differentiate, and integrate into particular neuronal systems (Clarke et al. 2000) (Fig. 3.4). Due to their therapeutic potential, NSCs have been a significant focus in AD research in the recent decades.

- NSCs in AD therapy and their therapeutic effects

In murine AD models, NSCs from healthy mice were transplanted into transgenic AD hippocampi. The study showed that NSCs could migrate, differentiate, and integrate into the neural circuits where the cognitive function of the AD mice was found to be improving 2 years after transplantation, as a result of an increase of cholinergic neuron numbers (Yamasaki et al. 2007). The first effect of NSC was

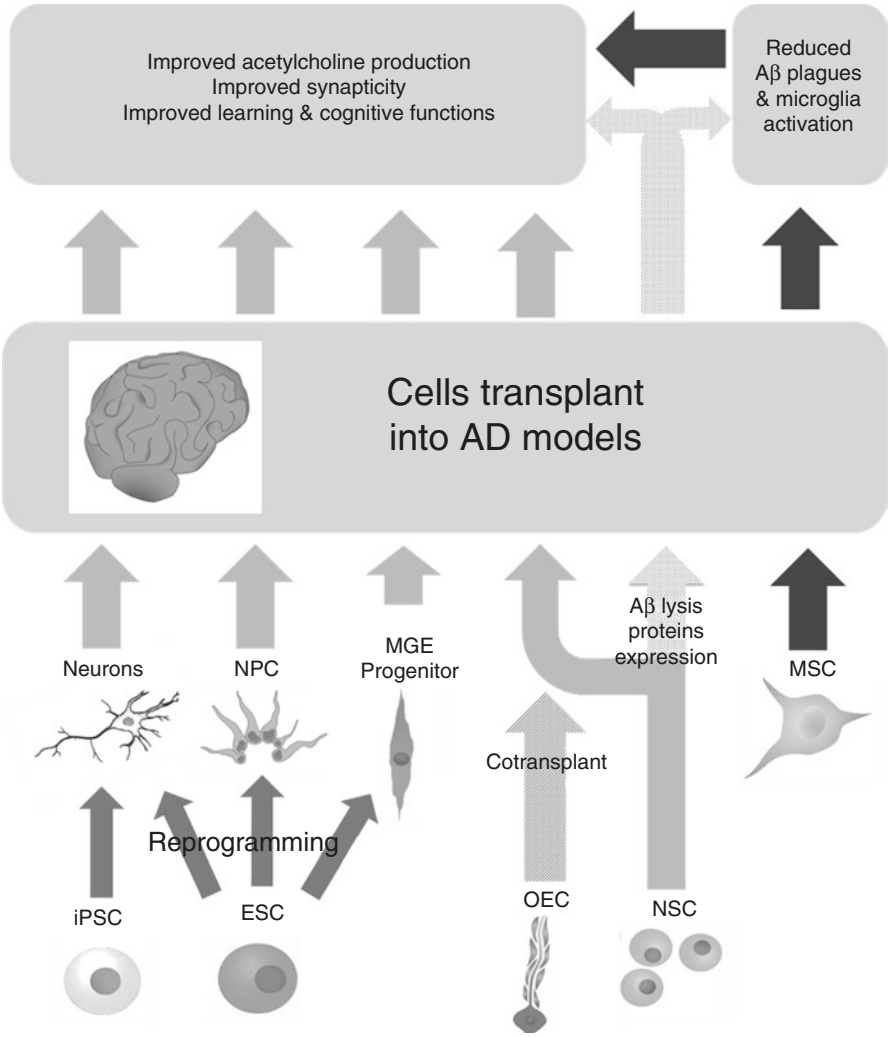

Fig. 3.4 Diagram summarizing different cell sources used for Alzheimer's disease therapy (Grskovic et al. 2011; Israel et al. 2012; Chen and Dong 2009; Marei et al. 2015; Kim et al. 2011; Brazel et al. 2003; Ma et al. 2013; Lee et al. 2009) (*Aβ* beta amyloid, *AD* Alzheimer's disease, *NPC* Neural precursor cells, *MGE* medial ganglionic eminence, *MSC* mesenchymal stem cells, *iPSC* induced pluripotent stem cells, *ESC* embryonic stem cells, *NSC* neural stem cells, *OEC* olfactory ensheathing cells)

believed to be its introduction of neurotrophin, a class of growth factor that induces survival, development, and functions of neurons. Neurotrophin is important in maintaining the strength and number of synapses which are crucial for synaptic plasticity and memory maintenance (Arancio and Chao 2007). Two main types of neurotrophin are brain-derived neurotrophic factor (BNDF) which is expressed in the cortex and the hippocampus, and nerve growth factor (NGF) which is also

expressed at the same regions and maintains survival and functions of cholinergic forebrain neurons (Kamei et al. 2007; Li et al. 2015; Lu et al. 2003). Neurotrophin production of NSCs is crucial in the treatment of AD, as it is shown that silencing of BDNF expression by shRNA in transplanted NSCs led to no improvement in synapse plasticity and cognitive functions (Blurton-Jones et al. 2009).

Other effects of NSCs in AD treatment were far less clear. NSCs were shown to improve endogenous neurogenesis and ischemia-induced axonal transport deficit in the cases of stroke (Jin et al. 2011). Even though reduction in neurogenesis and axonal transport deficit are highly present in AD, these effects of NSCs in AD are still uncertain. Moreover, NSCs also showed an anti-inflammatory effect by reducing migrogliosis and pro-inflammatory cytokines TNF-alpha production (Ryu et al. 2009). However, it is still not clear whether reducing inflammation produces a direct effect in the AD treatment.

- Genetic modifications in NSC therapy

NSCs could be genetically modified in different ways to enhance their efficiency in AD treatment. First, to improve their survival and proliferation in grafting, NSCs could be engineered to express higher levels of neurotrophin. For instance, human NSCs (hNSCs) derived from the 3–4 month-old fetal brain was engineered to express NGF or BDGF, which improves their survival and differentiation into neurons and astrocytes as well as memory restoration when transplanted into ibotenic acid-lesioned rat hippocampus (Marei et al. 2015). Moreover, as with acetylcholine production along with the decrease in cholinergic neuron numbers decrease in AD, NSCs could be engineered to express choline acetyltransferase (ChAT), the enzyme responsible for acetylecholine synthesis. A study showed that memory function was improved with an increase in the acetylcholine level after transplantation (Park et al. 2012b). The approach of genetically modified NSCs to release small therapeutic molecules was proven to be more effective than drug administration as NSCs could sustain the release of the molecules in a longer term, whereby they could localize at the regions affected by the disease and eliminate its efficacy loss as a result of inefficient transport through different barriers.

Even though NSCs could improve synaptic plasticity and cognitive function, they do not affect the Aβ plaque or Tau tangle pathology. As the level of Aβ may also influence the differentiation of NSCs, supplementing NSCs with Aβ therapeutic ability could provide long-term benefits. Genetically modifying NSCs to express neprilysin, an enzyme that degrades Aβ, is likely to be a potential method for AD treatment in the future (Kim et al. 2011) (Fig. 3.4).

- AD therapy using other types of stem cells

Besides NSCs, other stem cell types including neural precursor cells, embryonic stem cells (ESC), and mesenchymal stem cells (MSCs) could also be potential cell sources for AD therapy (Fig. 3.4).

Neural precursor cells, the cells that specifically differentiate into neurons, are found in embryonic medial ganglionic eminence (MGE), which is a transitory struc-

ture reside in the ventral ventricular zone of telencephalon where the progenitors originate and tangentially migrate during embryonic development (Brazel et al. 2003). These neural precursor cells are interneurons which produce acetylcholine as neurotransmitters and therefore have a role in the building of memory. It has been shown that MGE cells transplanted in the AD hippocampus could develop into mature interneurons, restoring memory and learning (Tong et al. 2014). Human ESCs could be treated with 1000 ng/ml of Sonic Hedgehog (SHH), causing the cells to differentiate into MGE-like cells. Upon transplanting into murine AD models, the ESC-derived MGE-like cells can differentiate into GABA cholinergic neuron and improve the host's cognitive function (Liu et al. 2013). Mouse ESCs could also be differentiated into NPC by the presence of growth factors such as NGF, SHH, and retinoic acid (RA). The cells then differentiated into cholinergic neurons when transplanted into the ibotenic acid-lesioned mice model, and showed improved memory recovery (Moghadam et al. 2009) (Fig. 3.4).

Apart from direct neural stem cell lines, MSCs, which are multipotent stromal cells that can differentiate into different cell types, were also shown to aid AD treatment. These MSC are derived from bone-marrow, human umbilical cord blood (Kim et al. 2012), and adipose tissues (Ma et al. 2013). For example, bone marrowed-derived MSCs were shown to reduce Aβ plaque deposition, tau hyperphosphorylation, and inflammation, as well as improving memory restoration in a transgenic murine AD model, where it was also suggested that the bone marrow derived MSCs could decrease Aβ plaque deposition. This suggestion leads to a belief that MSCs could contribute to AD therapy (Lee et al. 2009, 2010) (Fig. 3.4).

- Aiding stem cell therapy

In order to ensure successful neural restoration inside the brain, endogenous neurogenesis must also be justified to recover neural populations in the long term. One of the compounds that aid this process is allopregnanolone, which was shown to improve learning and cognitive function in triple mutated AD mice. Allopregnanolone increases the activation and proliferation of neural precursor cells as well as microglial cells, which are involved in neuroprotection (Wang et al. 2005). Another compound, fluoxetine, an anti-depressant, also promotes endogenous neurogenesis in the AD hippocampal model by inducing neural differentiation and neural protection in the presence of Aβ without glial differentiation (Chang et al. 2012).

Co-culturing NSCs grafted with other cell types could also promote survival and proliferation of neurons in the AD model. For example, NSCs could be co-transplanted with olfactory ensheathing cells (OECs) from fetal or adult olfactory bulb tissues. Such co-transplantation could promote the function of neurons significantly by improving the axonal transduction (Srivastava et al. 2009) (Fig. 3.4).

- Challenges in stem cell therapy of AD

Even though stem cell therapy has shown quite promising results in different studies, there has been a concern regarding immune rejection of the donor cells by the patient's tissues. The human leukocyte antigen haplotype of the donor cells must at least match with the recipients, who are required to take immunosuppression drug

to prevent the immune rejection (Chinen and Buckley 2010). The solution could potentially be the using of the patients' own NSCs which are limited in numbers, or iPSCs which may take relatively long time to successfully reprogramme and expand for the AD treatment. Other challenges may arise from the manufacturing of the stem cells in clinical use (Dunnett and Rosser 2014). These include variability of donor cells and their reprogramming methods, which may cause variations in their differentiation efficacy and clinical effects. Different clinical trials and control checkpoints must also be passed to ensure safe and ethical use of stem cell therapies and its commercial manufacture, which may take quite amount of time and budget to be completed.

3.6 Conclusion

Stem cell therapy, with the aids of its technological advances and the knowledge derived from the studies of stem cell models, has marked a great potential in the treatment of AD. Various experiments with animal models have proven its success. Nevertheless, there are different challenges to overcome before it could be clinically implemented in the future.

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Chapter 4
Stem Cell-Based Approaches for Treatment of Glioblastoma

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Abbreviations

WHO	World Health Organization
CBTRUS	Central Brain Tumor Registry of the United States
MGMT	O-6-methylguanine-DNA methyltransferase
NSC	Neural stem cell
OPC	Oligodendrocyte precursor cell
EGFR	Epidermal growth factor receptor
PTEN	Phosphatase tensin homolog
NF1	Neurofibromin 1
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
IDH1	Isocitrate dehydrogenase 1
GTR	Gross total resection
TMZ	Temozolomide
EBRT	External beam radiation therapy
RTK	Receptor tyrosine kinase
NSCLC	Non-small-cell lung carcinoma
mTOR	Mammalian target of rapamycin
TKI	Tyrosine kinase inhibitor

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MSC	Mesenchymal stem cell
5-FC	5-fluorocytosine
OV	Oncolytic virus
iPSC	Induced pluripotent stem cell
Glis1	Glis family zinc finger 1
ES cell	Embryonic stem cell
MADM	Mosaic analysis with double markers
shRNA	Small hairpin RNA
BBB	Blood-brain barrier
miRNA	MicroRNA
KLH	Keyhole limpet hemocyanin
CD::UPRT	Cytosine deaminase::uracil phosphoribosyltransferase
HDAC	Histone deacetylase inhibitor

4.1 Introduction

Glioblastoma (GB) is the most common and aggressive primary brain tumors in adults. A standard therapeutic approach for GB is maximal surgical resection followed by radiotherapy and adjuvant chemotherapy (Carlsson et al. 2014). Conventional treatments offer a modest increase in median survival in patients with GB, therefore the development of novel therapy is essential. Monoclonal antibodies and small-molecule inhibitors have been used in treatment of GB as novel targeted therapeutic agents (Prados et al. 2015). But, large number of different agents have failed to provide a significant improvement in survival of GB patients. Stem cells have been proposed as therapeutic tools for various diseases, including diabetes, neurodegenerative diseases, cardiovascular diseases, and cancer (Bovenberg et al. 2013). Stem cells have become attractive carriers due to their tumor tropic properties (Aboody et al. 2000). In this chapter, we review the current state of knowledge about stem cell based GB therapy.

4.2 Glioblastoma

4.2.1 Epidemiology

Glioblastoma is the most aggressive and prevalent primary brain tumors in adults (Ricard et al. 2012). Glioblastoma, previously called glioblastoma multiforme (due to high diversity in tumor size and shape), is the most frequent type of malignant gliomas (approximately 80%) (Omuro and DeAngelis 2013).

Glioblastoma multiforme (GBM), also known as grade IV GB, is the most common and aggressive form of Glioblastoma. Central Brain Tumor Registry of the

United States's (CBTRUS) reported that age-adjusted incidence of glioblastoma is 3.19/100,000 (Ostrom et al. 2013; Thakkar et al. 2014). Population-based studies have revealed that most of the tumors are located in the supratentorial region (Thakkar et al. 2014).

Glioblastoma has fatal outcome, and the mean duration of survival following diagnosis 12–15 months (Ostrom et al. 2013; Smoll et al. 2013). It has been shown that long-term survival in glioblastoma is associated with various clinical and biological parameters. In one of the pioneering population-based studies, Scott and colleagues have determined that younger age and lower Ki-67 values are associated with long-term survival, which is defined as survival equal to/exceeding 3 years (Scott et al. 1999). Later on, Krex and colleagues have found that long-term survivors are likely to have hypermethylation in the promoter region of O-6-methylguanine-DNA methyltransferase (*MGMT*) (Krex et al. 2007).

4.2.2 Genetic Background

The exact origin of glioma has been debated for a long time. Traditionally, glial cells of the CNS –mainly astrocytes and oligodendrocytes – have been considered as the potential origin of glioma. To address this controversial question, Liu and colleagues have used an ingenious technique called mosaic analysis with double markers (MADM) to trace cell lineages that originate from neural stem cells (NSCs) (Liu et al. 2011). The authors have identified that inactivation of neurofibromin (NF1) and p53 genes lead to pretransformation and hyperplasia in only oligodendrocyte precursor cells (OPCs), and not in NSCs (Liu et al. 2011). Moreover, they have determined that specific inactivation of these two tumor suppressor genes in OPCs also leads to formation of tumors, which are similar to NSC-derived tumors (Liu et al. 2011). These findings provide strong evidence for the potential origin of glioma, and emphasize the importance of a specific cellular origin that allows tumor formation.

The high level of heterogeneity in glioblastoma is not limited to morphological features, but also extend to molecular features. Comprehensive genomic and transcriptomic analyses have revealed the existence of four distinct molecular subtypes in glioblastoma: Neural, proneural, classical, and mesenchymal (Verhaak et al. 2010). Each subtype is defined by alterations in specific genetic signatures. For the classical subtype, the most common genetic alterations include amplification of chromosome 7, loss of chromosome 10, amplification of *EGFR* gene and corresponding overexpression of *EGFR* mRNA, point and/or vIII mutation in *EGFR*, homozygous deletion of *CDKN2A* (encodes for *p16INK4A* and *p14ARF*) and lack of *TP53* mutations (Verhaak et al. 2010). In case of mesenchymal subtype, the most characteristic alteration is the homozygous deletion of chromosome 17q11.2, which includes the *NF1* gene. Other alterations include comutations of *NF1* and phosphatase and tensin homolog (*PTEN*), and overexpression of genes involved in tumor necrosis factor (TNF) superfamily and NF-kB pathway (including *TRADD*, *RELB*,

Table 4.1 Overview of three dysregulated signaling pathways and their effector genes in glioblastoma

Pathway	Gene(s)	Type of alteration	Frequency (%)
PI(3)K/MAPK	*EGFR*	Amplification/mutation	57
	PDGFRA	Amplification/mutation	10
	MET	Amplification/mutation	1.6
	FGFR	Amplification/mutation	3.2
	PTEN	Deletion/mutation	41
	PIK(3)K	Amplification/mutation	25
	RAS	Amplification/mutation	1
	NF1	Deletion/mutation	10
	BRAF	Amplification/mutation	2
p53	*p53*	Deletion/mutation	28
	MDM2	Amplification/mutation	7.6
	MDM4	Amplification/mutation	7.2
Rb	*CDKN2A*	Deletion/mutation	61
	CDKN2B	Deletion/mutation	
	CDKN2C	Deletion/mutation	5.6
	CDK4	Amplification/mutation	14
	CDK6	Amplification/mutation	2
	Cyclins	Amplification/mutation	1.6
	RB1	Deletion/mutation	7.6

Adapted from Brennan et al. (2013)

TNFRSF1A) (Verhaak et al. 2010). The proneural subtype is characterized by amplification of chromosome 4q12 (including *PDGFRA* locus), and mutations in isocitrate dehydrogenase 1 (*IDH1*) gene (Verhaak et al. 2010). Additionally, high expression levels of oligodendrocytic development genes, including *NKX2-2* and *OLIG2*, are also common in this subtype (Verhaak et al. 2010).

Pathway analyses of these vast numbers of genomic alterations have revealed three most affected key signaling pathways (Brennan et al. 2013) (Table 4.1). Overall, PI(3)K/MAPK pathway is dysregulated in 90 % of cases, p53 pathway in 86 % of cases, and Rb pathway in 79 % of cases (Brennan et al. 2013).

4.3 Current Treatment Options for Glioblastoma

4.3.1 Standard of Care

The standard therapy for Glioblastoma is maximal surgical resection followed by radiotherapy and adjuvant temozolomide (TMZ) chemotherapy. The feasibility of surgical resection depends on tumor size and location (Carlsson et al. 2014). Global tumor resection is recommended, mainly because it reduces symptoms and increases survival (Orringer et al. 2012).

TMZ is a derivative of the dacarbazine, an alkylating agent, and exerts its antineoplastic activity by alkylating/methylating guanine (G) residues on the DNA backbone. By creating DNA damage, it interferes with normal DNA replication, and when left unrepaired, it leads to cell death. Adjuvant TMZ treatment after radiotherapy significantly improves survival of patients (Stupp et al. 2009). The benefit of TMZ treatment depends methylation status of MGMT gene, which abrogates the alkylating effect of TMZ. MGMT promoter methylation is a prognostic marker for TMZ response in Glioblastoma patients (Esteller et al. 2000; Hegi et al. 2005).

Radiotherapy aims to induce DNA damage (double-stranded breaks) to trigger apoptosis in tumor cells. In addition, radiosurgical methods, such as gamma knife, are used when the tumor size is relatively small (e.g. recurrent glioblastoma, when diagnosed early). Resistance to radiotherapy is quite common, and variant EGFR (EGFRvIII) is responsible from resistance to radiotherapy via inducing double-stranded DNA repair mechanisms (Mukherjee et al. 2009). In case of elder patients (>70 years), the effects of classic and accelerated radiotherapy are similar. Keime-Guibert and colleagues have reported that radiotherapy leads a modest increase in median survival in elderly patients with glioblastoma (Keime-Guibert et al. 2007).

4.3.2 Molecular Treatment Strategies

The current standard treatment of glioblastoma can only offer a modest increase in overall survival and life quality. Consequently, a broad range of molecular treatment strategies have been developed to overcome the therapeutic challenges, and to complement the standard of care. A comprehensive list of such agents are summarized in Table 4.2.

Targeted high-throughput screening studies have identified several small molecule inhibitors, which have therapeutic potential for treatment of glioblastoma. In one example, Trembath and colleagues have carried out a high-througput drug screening for EGFRvIII-expressing glioblastomas, and identified a compound (NSC-154829) which selectively inhibits the growth of glioblastoma cells expressing EGFRvIII (Trembath et al. 2007). In a recent study, Kitambi and colleagues have screened >1000 drugs in patient-derived glioblastoma cells, and identified a quinine-derivative (NSC13316; vacquinol-1) (Kitambi et al. 2014). The authors have found that vacquinol-1 treatment does not trigger cell death via classical mechanisms (apoptosis or autophagy), but rather through macropinocytosis (Kitambi et al. 2014). In addition, a small hairpin RNA (shRNA) screening has revealed that MAP kinase *MKK4* is responsible for conferring resistance to vacquinol-1-induced cell death (Kitambi et al. 2014). *In vivo* studies have shown that vacquinol-1treatment reduces tumor volume, and prolongs survival (Kitambi et al. 2014). Given its ability to cross the blood brain barrier (BBB), and significant antitumor activity, vacquinol-1 appears as a promising therapeutic molecule for treatment of glioblastoma.

EGFRvIII is also a preferred molecular target for immunotherapy strategies. A promising example is rindopepimut, a therapeutic agent that consists of a short,

Table 4.2 Available molecular targeted therapy options for glioblastoma.

Therapeutic agent	Type of tumor	Status	References
Gefitinib (ZD1839)	Recurrent glioblastoma	Phase I/II	Rich et al. (2004)
Erlotinib (OSI-774)	Glioblastoma Recurrent glioblastoma	Phase I/II	van den Bent et al. (2009) Raizer et al. (2010) Prados et al. (2006) Peereboom et al. (2010)
Lapatinib (GW-572016)	Glioblastoma	Phase I/II	Thiessen et al. (2010)
Cetuximab (C225)	Recurrent high-grade glioma	Phase II	Neyns et al. (2009)
ZD6474	Glioblastoma	Preclinical studies	Damiano et al. (2005) Jo et al. (2012) Shen et al. (2013)
Imatinib mesylate (STI571)	Recurrent glioblastoma	Phase II	Reardon et al. (2005) Reardon et al. (2005)
Sunitinib (SUO11248)	Recurrent glioblastoma	Phase II Preclinical studies	Neyns et al. (2011) Kreisl et al. (2013)
Vandetanib (PTK787)	Recurrent malignant glioma	Phase I/II	Kreisl et al. (2012)
Vatalanib	Recurrent glioblastoma	Phase I/II	Gerstner et al. (2011)
Sorafenib	Recurrent glioblastoma	Phase I/II	Galanis et al. (2013) Nabors et al. (2011)
AZD2171	Glioblastoma	Phase II	Batchelor et al. (2007)
Endostatin	Glioblastoma	Preclinical studies	Barnett et al. (2004)
Angiostatin	Malignant glioma	Preclinical studies	Kirsch et al. (1998)
Atrasentan	Recurrent glioblastoma	Phase I	Phuphanich et al. (2008)
Rindopepimut	Newly diagnosed glioblastoma Relapsed glioblastoma	Phase III Phase II	Ongoing (NCT01480479) Ongoing (NCT01498328)
Vorinostat	Recurrent glioblastoma	Phase II	Galanis et al. (2009)
Romidepsin (FK228, depsipeptide)	Recurrent malignant glioma	Phase I/II	Iwamoto et al. (2011)
Valproic acid	Glioblastoma	Phase II	Ongoing (NCT00302159)
Bortezomib (PS-341)	Recurrent malignant glioma	Phase I	Phuphanich et al. (2010)
MG-132	Glioblastoma	Preclinical studies	Zanotto-Filho et al. (2012) Shimizu et al. (2013) Fan et al. (2011)

Table 4.2 (continued)

Therapeutic agent	Type of tumor	Status	References
UCN-01	Glioblastoma	Preclinical studies	Meng et al. (2005) Witham et al. (2002)
Flavopiridol	Glioblastoma	Preclinical studies	Hayashi et al. (2013) Newcomb et al. (2003) Alonso et al. (2003)
O6-benzylguanine	Recurrent glioblastoma	Phase I Phase II	Quinn et al. (2009a) Quinn et al. (2009b)
Olaparib (AZD2281)	Relapsed glioblastoma	Phase I	Ongoing (NCT01390571)
Aldoxorubicin	Glioblastoma	Phase II	Ongoing (NCT02014844)
AR-67	Recurrent glioblastoma	Phase II	Ongoing (NCT01124539)

EGFRvIII-specific peptide coupled to a carrier protein (keyhole limpet hemocyanin; KLH). Preclinical studies have shown that rindopepimut exerts its antitumor effects by eliciting humoral and cellular responses (Babu and Adamson 2012). Rindopepimut is currently being tested in Phase II trial (for relapsed glioblastoma; together with granulocyte colony stimulating factor) and in Phase III trial (for newly diagnosed glioblastoma).

Mutations in *IDH1* are quite common (>80 %) in secondary glioblastoma (Verhaak et al. 2010; Kloosterhof et al. 2011). In addition, it has been shown that *IDH1*mutations are one of the earliest genetic alterations in development of astrocytomas (Watanabe et al. 2009). These findings suggest that *IDH1*could be a therapeutic target for glioblastoma. Indeed, Rohle and colleagues have identified a specific inhibitor of mutant *IDH1*, and found that inhibitor treatment impairs the growth of glioblastoma cells harboring mutant *IDH1*, while cells with wild-type *IDH1* are not effected (Rohle et al. 2013).

4.3.3 Limitations of Available Treatments

Molecular targeted therapies involving single agents have failed to provide a breakthrough for treatment of glioblastoma, due to several biological and technical reasons (Table 4.3).

Coactivation of receptor tyrosine kinases (RTKs) is a common event in different cancer types (Stommel et al. 2007). This phenomenon might explain the limited efficacy of single-targeted treatment strategies in glioblastoma. Another possibility is the activation of alternative RTKs as a “compensation mechanism”. Apart from these mechanisms, tumor cells could also become resistant to treatment, as a result of secondary mutations in the target molecule such as EGFR (Giaccone 2005; Shih et al. 2005). In either case, the ultimate result is the limited efficacy of single-targeted treatment.

Table 4.3 Major limitations of available treatment strategies for glioblastoma

Limitation	Reason
Intratumoral heterogeneity	Molecular subtypes of glioblastoma respond differently to treatment
Tumor evolution	Emergence of compensation mechanisms (e.g. activation of other signaling pathways in response to TKIs)
Inadequate preclinical models	*In vitro* and *in vivo* models do not fully reflect the clinical settings
Insufficient tumor specificity	Severe side effects in nontumorigenic cells and/or tissues
Low concentration of therapeutic agent in tumor	Low penetrability; clearance/inactivation mechanisms; stability in circulation

Tumor evolution is another issue in terms of chemoresistance. Chemotherapy is involved in tumor evolution through acquisition of new mutations (Prados et al. 2015). Johnson and colleagues have reported that TMZ treatment causes progression of glioblastoma via hypermutation in RB and AKT/mTOR signaling pathways (Johnson et al. 2014).

Glioblastoma is a highly heterogeneous tumor, and the existence of distinct molecular subtypes might explain why a large number of different agents have failed to provide a significant improvement in survival of glioblastoma patients. Development and/or identification of novel therapeutic agents that are specific for a given molecular subtype has the potential to overcome this problem.

4.4 Application of Stem Cells to Glioblastoma Therapy

4.4.1 Rationale for Stem Cell-Based Therapies

An exceptional feature of stem/progenitor cells with neural or mesenchymal origin is their inherent tumor tropism (Aboody et al. 2000; Benedetti et al. 2000). Glioma cells release several cytokines, including hepatocyte growth factor (HGF), stromal cell derived factor 1 (SDF-1), urokinase-type plasminogen activator (uPA), vascular endothelial growth factor (VEGF) (Schmidt et al. 2005), and it is hypothesized that this cytokine gradient is responsible for attracting NSCs (Zhao et al. 2008). In another study, Hata and colleagues have shown that platelet-derived growth factor BB (PDGF-BB) is responsible, in part, for the tropism of bone marrow-derived MSCs to glioma (Hata et al. 2010). This unique feature make NSCs and/or MSCs ideal delivery tools for cancer treatment, as they can be exploited to achieve great treatment specificity.

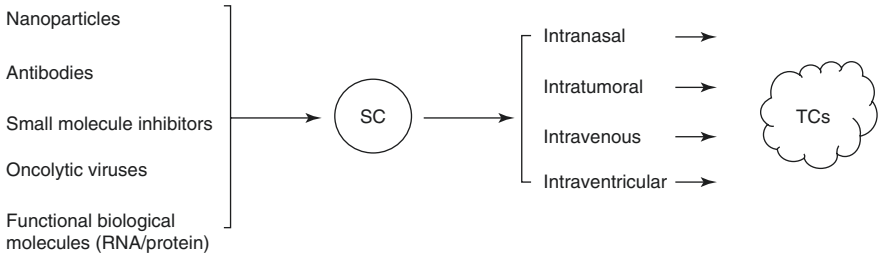

Fig. 4.1 Overview of stem cell-based therapeutic strategies for treatment of glioblastoma. Stem cells can be modified to express and/or to deliver different classes of therapeutic agents. These include nanoparticles, oncolytic viruses, functional biological molecules (RNA or protein species), antibodies, and small molecule inhibitors. Afterwards, the modified stem cells can be administered through different routes

4.4.2 Application of Stem Cells for Treatment of Glioblastoma

Stem cells can be exploited for cancer treatment in different ways. One approach involves the genetic modification of stem cells, so that they express cytotoxic molecules (e.g. antiangiogenic proteins, apoptotic molecules, immunomodulatory cytokines etc.). Alternatively, stem cells can be used as "vehicles" to deliver therapeutic molecules (e.g. oncolytic viruses, nanoparticles, small molecule inhibitors etc.) (Fig. 4.1).

PTEN, a well-characterized tumor suppressor gene that is frequently mutated and/or deleted in different cancer types, is the major negative regulator of the PI3K/Akt signaling pathway (Song et al. 2012). In addition, it is known that a significant portion of glioblastoma tumors have mutations and/or deletions in *PTEN*. Thus, reintroduction of PTEN expression could interfere with glioblastoma tumor growth. Dasari and colleagues have cocultured glioblastoma cell lines with human umbilical cord-derived MSCs, and characterized the downstream cellular effects. The authors have found upregulation of PTEN expression, as well as downregulation of several downstream molecules (including Akt, JUN, PI3K, RAS, RAF1), which results in inhibition of glioblastoma cell migration (Dasari et al. 2010).

Angiogenesis is defined as the process of new blood vessel formation from preexisting ones. This process is not only essential for normal development, but also for tumor cells. Certain molecules (known as *proangiogenic factors*) induce angiogenesis through the action of endothelial cells and endothelial progenitor cells (Ho et al. 2013). The well-known proangiogenic factors include VEGF, fibroblast growth factor (FGF), PDGF/PDGR, angiopoetin 1 and 2 (Ang1 and Ang2). In addition, different studies have shown that VEGF and PDGF/PDGR axis play key roles in glioblastoma angiogenesis (Jain et al. 2007; Das and Marsden 2013). Given its vital role for tumor sustainability, angiogenesis is considered as a potential target

for treatment of glioblastoma. Ho and colleagues have demonstrated that human bone marrow-derived MSCs attenuate glioblastoma tumor growth through the inhibition of PDGF/PDGR axis (Ho et al. 2013).

Benedetti and colleagues have modified neural precursor cells to express interleukin 4 (IL-4) gene, and injected the cells into syngeneic brain glioblastoma in mice (Benedetti et al. 2000). Their results indicate that this treatment leads to survival of most mice (Benedetti et al. 2000).

4.4.3 Immunotoxins

Another treatment strategy involves the delivery of immunotoxins. These fusion proteins consist of two parts: A targeting residue (usually a monoclonal antibody or a growth factor against a specific molecular marker on tumor cells) and a cytotoxic molecule (such as Diptheria toxin or Pseudomonas exotoxin). In 2011, Sun and colleagues have demonstrated that human bone marrow-derived MSCs could be engineered to express an EphA2-specific immunotoxin, and these MSCs selectively kill glioblastoma cells *in vitro* and *in vivo* (Sun et al. 2011).

PEX is a part of the human metalloproteinase-2, and is overexpressed in glioblastoma (Bello et al. 2001). It has been shown that PEX inhibits angiogenesis, cell proliferation, and migration in glioblastoma (Brooks et al. 1998; Bello et al. 2001). Based on these findings, Kim and colleagues have introduced PEX gene in human NSCs, and used tested modified NSCs in a mouse model of glioblastoma. Their results have shown that PEX-containing NSCs effectively suppressed angiogenesis, cell proliferation, and tumor growth (Brooks et al. 1998).

4.4.4 Suicide Gene Fusion

The cytosine deaminase::uracil phosphoribosyltransferase/5-fluorocytosine (CD::UPRT/5-FC) system is one of the best characterized suicide gene/prodrug systems for cancer treatment.

In a preclinical mouse model, Aboody and colleagues have tested whether CD-expressing NSCs could be a successful strategy for treatment of glioblastoma (Aboody et al. 2013). *In vitro* characterization studies showed that modified NSCs exhibit normal karyotype and tumor tropism, and the authors found that combination therapy with CD-expressing NSCs and 5-FC caused a significant decrease in tumor volume (>60 %) (Aboody et al. 2013). Overall, these results confirm the safety and efficacy of the method, and this study has led to the approval of a first-in-human study in patients with recurrent high-grade glioma.

In another study, Altaner and colleagues have engineered human bone marrow-derived MSCs to express CD::UPRT, and injected these therapeutic MSCs into the resection cavity. The authors have administered the prodrug via

continuous intraventricular injections. Their results have shown that MSC-mediated suicide gene/prodrug strategy leads to permanent complete regression and/or curative therapy (depending on the application scheme) in treated animals (Altaner et al. 2014).

4.4.5 Oncolytic Viruses

Viruses, which preferentially infect tumor cells and induce lysis as a result of their replication, are known as oncolytic viruses (OVs) (Nemunaitis 1999). These viruses can be engineered in different ways, so that they replicate only in tumor cells. Thus, OVs can be exploited as therapeutic tools for cancer treatment.

Delta-24-RGD is a type of oncolytic virus, which is capable of replicating only in tumor cells that contain an inactive retinoblastoma protein. Yong and colleagues have loaded this virus into human bone marrow-derived MSCs, and tested the efficacy of intravascular delivery in a mouse model of glioblastoma (Yong et al. 2009). The authors have found a significant improvement in median survival (75.5 days vs 42 days) in the treated mice, as well as tumor eradication in certain subset of animals (Yong et al. 2009).

In a recent study, Ahmed and colleagues have loaded an oncolytic adenovirus (CRAd-Survivin-pk7) into a FDA-approved NSC line (HB1.F3.CD), and tested the efficacy of this treatment strategy in mouse models of glioblastoma (Ahmed et al. 2013). The authors have found a significant improvement in median survival in mice treated with OV-loaded NSCs (Ahmed et al. 2013). In addition, their results have shown that OV-loaded NSCs are nontumorigenic *in vivo* (Ahmed et al. 2013). Taken together, the authors have concluded that OV-loaded NSCs should be tested in a Phase I clinical trial in patients with glioblastoma.

Despite their limited therapeutic efficacy, antibodies have appeared as promising agents for treatment of different cancers. Stem cells can be modified to express antibodies of interest (as whole or single chain), and can be used to suppress tumor growth (Young et al. 2014). EGFRvIII is a tumor-specific antigen for glioblastoma, and can be used to target tumor cells. Balyasnikova and colleagues have modified MSCs with a single-chain antibody against EGFRvIII, and tested the therapeutic potential of this strategy *in vitro* and *in vivo*. Notably, the authors have found that modified MSCs results in 50% reduction in tumor growth, and a decrease in the density of CD31-positive blood vessels (Balyasnikova et al. 2010).

4.4.6 miRNAs

MicroRNAs (miRNA) are small, noncoding RNAs with diverse roles in normal homeostasis and disease states. Alterations in miRNA expression levels have been identified virtually in all types of cancers, including glioblastoma. In addition, it has been demonstrated that miRNA signatures characterize distinct molecular subtypes

in glioblastoma, and may contribute to tumor pathogenesis (Kim et al. 2011). Thus, miRNAs have potential to serve as therapeutic targets and/or molecules for treatment of glioblastoma. Recently, Lee and colleagues have shown that bone marrow-derived MSCs are able to deliver synthetic miRNA mimics to glioblastoma cells *in vitro* and *in vivo* (Lee et al. 2013). In addition, their results indicate that MSC-mediated delivery of miRNA mimics inhibits cell migration and self-renewal ability of glioblastoma stem cells (Lee et al. 2013).

4.5 Future Directions

4.5.1 Limitations and Potential Solutions

The inherent tumor tropism of different types of stem cells make them ideal candidate for therapeutic applications. Characterization studies have shown that certain molecules (growth factors, hypoxia-induced factors, chemokines etc.) are crucial for this "homing mechanism" (Zhao et al. 2008; Bovenberg et al. 2013). Further understanding of how tumors affect their microenvironment – especially by analyzing the tumor "secretome" – will help us to identify novel molecules which could regulate tumor tropism. Such studies will provide the necessary information to enhance the tumor tropism of stem cells.

Different strategies can be employed to improve the efficacy of NSC-based therapeutics. Morshed and colleagues have suggested that stimulating key receptors and/or signaling pathways involved in tumor tropism might improve the distribution of NSCs (Morshed et al. 2015).

Preclinical studies involving intranasal administration of stem cells are currently in an early stage; this method have certain advantages over other delivery routes. The main advantage of intranasal administration is the rapid cell migration to the target area. Different groups have demonstrated that cell migration (in case of NSCs) occurs rapidly in case of intranasal administration (1–2 h), compared to intravenous administration (10–20 days) (Wu et al. 2013). Peripheral injection of NSCs results in transient colonization of the systemic organs; on the other hand, in case of intranasal administration, NSCs migrate directly into the CNS (Wu et al. 2013). Moreover, due to its noninvasive nature, intranasal administration can be repeated multiple times.

The success of systemic stem cell delivery depends on the ability of stem cells to cross the BBB. Owing to the presence of tight junctions and efflux transporters (e.g. P-glycoprotein), this anatomical barrier has been considered as the major obstacle that limits the efficacy of systemic delivery strategies for treatment of glioblastoma. Recent studies, on the other hand, have challenged this notion and suggested that BBB has "heterogeneous integrity" – i.e. disrupted at/near core region, intact along the edges (Agarwal et al. 2013; van Tellingen et al. 2015). Supporting evidence for successful systemic delivery of stem cells have come from *in vivo* studies, which

have demonstrated that NSCs and MSCs are able to migrate to intracranial tumors in mouse models of glioblastoma (Aboody et al. 2000; Nakamizo et al. 2005; Watkins et al. 2014). Still, it is clear that optimization of existing strategies and/or development of novel strategies warrants further understanding of the integrity of BBB, and intercellular interactions in the BBB microenvironment.

Another important point to consider is the "fate" of the NSCs/MSCs in the tumor microenvironment. The transdifferentiation ability of bone marrow-derived MSCs provide them with a dual function; once differentiated into activated fibroblasts or endothelial-/pericyte-like cells, these cells can support tumor growth. On the contrary, other studies provide evidence for tumor suppressive function of these cells. This phenomenon is discussed in detail in a recent review (Barcellos-de-Souza et al. 2013).

4.5.2 Ethical Concerns

Throughout the world, research on stem cells – especially embryonic stem cells (ES) – has been tightly linked to ethical and social controversies. Undoubtedly, the major concern has involved the moral question of destructing embryos, as this represents the initial approach to harvest ES cells. The main argument against research on ES cells is regarding the potential of embryos to become human beings; thus, the opponents of ES cell search strongly claim that embryos should have the same moral status as adults (Lo and Parham 2009).

The year 2006 represents a milestone in stem cell research. A Japanese research group has reported the possibility of obtaining induced pluripotent stem cells (iPSCs) from mouse embryonic and adult fibroblasts by addition of four defined transcription factors, namely Oct3/4, Sox2, Klf4, c-Myc (Takahashi and Yamanaka 2006). This groundbreaking discovery was quickly followed by two other reports, which demonstrate the possibility to generate ES-like cells by introducing the same set of transcription factors into human fibroblasts (Takahashi et al. 2007; Wernig et al. 2007).

These promising results have suggested that iPSCs could "substitute" for ES cells. However, certain technical challenges related to iPSC technology should be overcome before removing ES cells from the picture. First, the initial methods heavily relied on retroviral transduction of reprogramming factors, including c-Myc oncogene. The use of retroviruses and oncogenes has raised serious concerns about the safety of this protocol, and is considered as a limiting factor for their application to clinical settings. It has been reported that 25 % of mice, which are transplanted with iPSCs harboring c-Myc, develop lethal teratomas. To overcome this limitation, other members of the Myc protein family (N-Myc and I-Myc) and Glis family zinc finger 1 (Glis1) have been tested as alternative reprogramming factors (Maekawa et al. 2011). Despite the promising results, future preclinical studies are necessary to test

NSCs are traditionally derived from ES cells, which raise ethical concerns. The development of iPSCs has offered an alternative solution to this problem; however, this field is still in its infancy, and comprehensive studies are required to test the safety of the method in preclinical and clinical settings. Direct reprogramming (or direct conversion) with predefined factors can be used to obtain stable NSCs from fibroblasts (Han et al. 2012; Thier et al. 2012). Thus, this method can be employed as a safer and faster alternative, compared to the iPSC technique.

4.5.3 Concluding Remarks

During the past decade, several studies have been directed towards molecular profiling of glioblastoma. These efforts have significantly increased our understanding of this deadly tumor, and given rise to the development novel therapeutic strategies. Still, glioblastoma continues to be a major cause of cancer-associated mortality throughout the world. The recent advances in the field of stem cells have made it possible to employ stem cells as superior therapeutic tools for treatment of different cancers, including glioblastoma. The efficacy and safety of stem cell-based treatment strategies awaits comprehensive preclinical and clinical studies. Only time will tell whether stem cells will finally offer a "lasting" solution for this devastating cancer.

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Chapter 5
Stem Cell-Based Therapies for Parkinson's Disease

Charlotte Palmer and Isabel Liste

5.1 Introduction

Parkinson's disease (PD) is a chronic neurodegenerative disorder that is especially common in the elderly. It results from the progressive death of pigmented dopaminergic neurons (DAn) in the *substantia nigra pars compacta* (SNpc) that project axons to the striatum where dopamine is released (Buttery and Barker 2014; Martínez-Morales and Liste 2012; Lindvall and Kokaia 2009). This provokes a reduction in striatal dopamine levels, causing most of the clinical motor symptoms, which include tremors, rigidity, bradykinesia and other debilitating symptoms (Savitt et al. 2006).

It is now known that the pathology extend far beyond the nigrostriatal dopaminergic pathway itself, as other dopaminergic and non-dopaminergic systems are also affected in PD. Accordingly, in addition to the better-known motor symptoms, PD patients suffer several nonmotor symptoms. These symptoms can include sleep disturbances, dementia and mood disturbances which can have a significant impact on the quality of life of patients (Lindvall 2016).

The etiology of PD is still not fully understood. However, some possible pathogenic mechanisms have been proposed such as impairment of mitochondrial function and trophic support, abnormal action of kinases, excessive release of oxygen free radicals and dysfunction of protein degradation (Wang et al. 2015).

Besides death of DAn, other hallmarks of PD include the presence of protein aggregates made up of α-synuclein-positive Lewy bodies in several brain regions, as well as neuro-inflammation causing the disease progression (Wang et al. 2015; More et al. 2013; McGeer and McGeer 2008). Indeed, activation of microglial and glial cells and inflammatory responses are common features of both animal models

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of PD and PD patients, playing a significant role in the neurodegenerative progression of DAn (Wang et al. 2015; Hirsch et al. 2012).

Today, there is still no cure for PD. However, a variety of treatment options are available to help relieve motor symptoms, which can greatly improve the quality of life of the patients.

Current treatments include: deep brain stimulation (DBS) in the subthalamic nucleus or globus pallidus. This treatment option **co**nsists of placing a stereotactically guided microelectrode into a specific brain structure. A stimulator is also implanted into the chest of the patient, adjusting the stimulation parameters by telemetry. DBS is a safe and reversible procedure that modulates specific targets in the brain resulting in motor symptom improvement. DBS is especially effective managing long-term motor complications resulting from L-dopa treatment, such as dyskinesias and wearing-off phenomena. However, the technique still needs to be optimized in order to increase efficiency, as the procedure is expensive and not all patients are equally likely to improve (Larson 2014; Heumann et al. 2014)

Available pharmacological therapies aim to increase dopamine levels in the brain by providing dopaminergic agonists, or inhiting dopamine breakdown (catechol-O-methyl transferase and monoamine oxidase inhibitors) (Lindvall 2016; Prashanth et al. 2011). The most common treatment for PD is levodopa (L-dopa). L-dopa is able to cross the blood brain barrier and once in the brain is transformed into dopamine by dopaminergic neurons.

Previously described treatments are effective in alleviating some symptoms during early phases of the disease, but long-term efficacy is unknown. Furthermore, these treatments are associated with certain side effects, including motor fluctuations, on-off phenomena and involuntary movements as dyskinesias. Ultimately, none of these treatments are reparative, and do not stop the disease from progressing (Lindvall 2016; Poewe 2009; Politis et al. 2014).

Cell-replacement clinical trials based on transplantation of human fetal mesencephalic tissue, a tissue rich in dopaminergic neuroblasts, have provided *proof of principle* that cell replacement therapy can work in the human PD brain (Barker et al. 2013; Björklund and Dunnett 2007). In the most successful trials, DAn generated from the transplanted tissue was able to re-innervate the denervated striatum and become functionally integrated, restoring the striatal DA release and giving rise to clear symptomatic relief in some patients (Kefalopoulou et al. 2014; Petit et al. 2014; Piccini et al. 1999).

However, ethical and practical aspects related to tissue availability limit their widespread clinical use. It is therefore necessary to seek alternative cell sources, mainly based on the use of stem cells. Due to their properties, stem cells are now considered best candidates as an alternative to DAn, different from fetal mesencephalic tissue.

Human DA precursors have been efficiently derived from different sources of stem cells including: human Embryonic Stem Cells (hESCs) (Kriks et al. 2011; Cho et al. 2008; Chambers et al. 2009; Malmersjö et al. 2010), human induced Pluripotent Stem Cells (hiPSCs) isolated from control, or from PD patients (Soldner et al. 2009; Hargus et al. 2010; Nguyen et al. 2011; Sánchez-Danés et al. 2012), human Neural Stem Cells (hNSCs) both from fetal (Courtois et al. 2010; Villa et al. 2009) or adult

brains (Lévesque et al. 2009) human Mesenchymal Stem Cells (hMSCs) by the induction with different cytokines and neurotrophic factors like GDNF (Kitada and Dezawa 2012; Trzaska and Rameshwar 2011; Dezawa et al. 2004). More recently, it has been shown the direct conversion of fibroblasts into functional "induced" DAn (iDA). Still has to be explored to what extent these cells can contribute to functional recovery in models of PD (Caiazzo et al. 2011; Pfisterer et al. 2011).

In this chapter, we discuss some general issues related to the clinical use of stem cells to treat Parkinson's disease. We describe the different types of stem cells available nowadays, their properties and how they are being developed and applied in PD patients.

5.2 Grafts of Human Fetal Ventral Mesencephalic Tissue

The initial idea for cell transplantation in PD was simple: in theory, adult DAn lost by neurodegenerative processes could be replaced by immature human DAn (Brundin et al. 1988).

It all started between the 1970s-1980s, several different groups demonstrated that DAn obtained from the fetal ventral mesencephalon (VM) were able to survive and integrate into the host tissue, release dopamine and enhance motor function in animal models of PD.

Similar results were obtained with mesencephalic xenografts transplanted in the striatum of rats under immunosuppression (Brundin et al. 1988). The promising results from these experimental trials then allowed several groups to perform open label clinical trials in PD patients.

Cell replacement therapies (CRT) for PD have been conducted during the last 30 years using different source of cells. The most effective cells have so far been allogenic fetal ventral mesencephalic tissue grafts, which contains developing midbrain DAn and their precursors.

In general transplants are done in the striatum, the region where project their axons dopaminergic neurons of SNpc. Successful open-label trials reported improved motor symptoms in a number of patients (Barker et al. 2013; Björklund and Dunnett 2007; Freed et al. 1992; Spencer et al. 1992; Widner et al. 1992; Lindvall et al. 1990), improved ^{18}F –DOPA uptake (Piccini et al. 1999; Lindvall et al. 1994; Peschanski et al. 1994) and robust long-term graft survival lasting over a decade as shown by postmortem analysis, even though some grafted cells showed Lewy body formation (Hallett et al. 2014; Kordower et al. 2008; Li et al. 2010).

Additionally, the grafted tissue re-innervated the host striatum and became functionally integrated into the recipient circuitry (Kefalopoulou et al. 2014; Petit et al. 2014; Piccini et al. 1999). However, two placebo-controlled trials showed modest benefit in the first end point analyzed (Freed et al. 2001; Olanow et al. 2003). Furthermore a group of patients developed graft-induced dyskinesia that persisted even in the absence of L-dopa treatment (Hagell et al. 2002). Fortunately, a later evaluation of these grafts by F-DOPA uptake and UPDRS scores, showed statistically significant improvement of fetal tissue transplantation for certain patients, in line with reports from open-label trials (Barker et al. 2013; Freed et al. 2011).

The reasons behind the inconsistency of these results may be related to poor standardization procedures, including patient selection, tissue preparation, immunosuppression procedures, primary endpoints, and general trial design. In any case, all these trials have revealed several limitations of the procedure for routine clinical practice that should be improved in order to develop a viable cell replacement therapy (CRT) for PD (Olanow et al. 2003; Isacson et al. 2003; Ganz et al. 2011). The main challenges are related to ethical issues related to the use of fetal tissue, poor standardization of the tissue dissection and cell material processing. This last limitation can contribute to the appearance of dyskinesia (related in part, to the serotonergic component of the graft), be related to the high variability in graft survival and be associated with inconsistent clinical benefit (Carta et al. 2008; Politis et al. 2010). Another important limitation is the host immunological and inflammatory response, since autologous tissue cannot be used (Rath et al. 2013; Piquet et al. 2012; Arenas 2010; Piccini et al. 2005).

There are several problems associated with performing such transplants. First of all it is extremely difficult to obtain sufficient amounts of fetal tissue, as each patient requires tissue obtained from between 4 and 10 aborted fetuses that must also have the adequate embryonic age. Currently there is no good method of cryopreservation; the mesencephalic tissue can be maintained at 4 °C for 1 week, but the quality of the tissue decreases with longer storage times. Furthermore, due to the need to mix cell suspensions from different donors, it is complicated to control HLA system; therefore it is difficult to standardize the quality of the donor cells.

Currently, the European consortium TRANSEURO (www.transeuro.org.uk) (see Table 5.1) is making significant efforts to optimize the design of clinical trials for CRT in PD. The main objective of this project is to develop a safe and effective method for treating PD patients using fetal VM cells that can serve as a model for future clinical trials.

This trial is designed to minimize technical variables such as: patient selection (age, type of PD), tissue preparation and collection, graft placement and support, immunosuppressive treatment, follow up time and quantifiable endpoints. Therefore, this study can serve as a reference to develop future stem-cell transplantation assays and will provide a more coherent view of the current therapeutic improvement (Abbott 2014; Moore et al. 2014; Gonzalez et al. 2015a).

5.3 Stem Cell Properties and Requirements for Their Application in CRT for PD

As mentioned above, a major challenge in developing cell transplantation into a routine clinical practice for PD is the deficiency of fetal tissue supply, which implies the use of several fetal tissue to treat a single patient. For this reason, important research efforts have been carried out to find alternative sources of cells for

Table 5.1 Human stem cells used in clinical trials for treatment of Parkinson's disease

Stem cell type	Transplant type	Delivery administration	Status	Sponsor
hMSCs from bone marrow	Allogenic	Intravenous administration	Phase 1 NCT02611167	The University of Texas Health Science Center
	Autologous	Intravenous administration	Phase 1/2 NCT01446614	Guangzhou General Hospital of Guangzhou Military Command
Adipose-Derived hMSCs	Autologous	into the Vertebral Artery and Intravenously	Phase 1/2 NCT01453803	Ageless Regenerative Institute
	Autologous	Not provided	Recruiting NCT02184546	StemGenex
hNSCs from fetal ventral mesencephalic tissue	Allogenic	Intracerebral implantation	Phase 1 (TransEuro Project) NCT01898390	University of Cambridge
	Allogenic	Not provided	Phase 1/2 NCT01860794	Bundang CHA Hospital
	Allogenic	Intracerebral implantation	NCT02538315	University of Saskatchewan
hNSCs from adult cerebral cortex	Autologous	Intracerebral implantation to the left putamen	Phase 0 NCT01329926	NeuroGeneration (Lévesque et al. 2009)
Human parthenogenetic-derived NSCs	Allogenic	Intracerebral implantation to the striatum and Substantia Nigra	Phase 1 NCT02452723	Cyto Therapeutics Pty Limited

Abbreviations: *hMSCs* human mesenchymal stem cells, *hNSCs* human neural stem cells

transplantation in PD. Several cell sources have been explored in order to generate DAn. The most promising cells found so far are stem cells.

Stem cells are undifferentiated cells characterized by their ability to proliferate and differentiate into more specialized types of cells. Stem cells can be classified according to how they were obtained or by their differentiation potential. Based on their ability to differentiate, stem cells are divided basically into two major categories: pluripotent stem cells (which can give rise to specialized cells of the three germ layers, i.e. endoderm, mesoderm and ectoderm) and multipotent stem cells (more specialized cells, that can generate specific cell lineages of a particular germ layer, although recently it has been shown that some multipotent cells possess the capacity to transdifferentiate into cells of more than one germ layer, such as MSCs) (Bongso et al. 2008; Zhan and Kilian 2013; Macias et al. 2010).

Overall it is assumed that in order to make the differentiation of DAn from stem cells a clinically competitive treatment option for PD, these cells need to be equivalent to those of human VM tissue in terms of their phenotype, as well as neurochemical and electrophysiological properties both *in vitro* and *in vivo* after grafting.

This means that transplanted cells must induce a substantial improvement of motor symptoms, without causing side effects (Lindvall et al. 2012; Martínez-Serrano and Liste 2010). To achieve this, grafted cells must survive, re-innervate the striatum, integrate into the neural circuitry of the host and exhibit the same characteristics of authentic nigral A9 DAn.

Also they have to satisfy a number of safety requirements such as not forming tumors, avoiding the development of dyskinesia, either by the presence of serotonergic neurons or inappropriate distribution of implants, and they should not induce immune rejection in the host. Furthermore, it must be possible to grow sufficient numbers of these cells in order to reach clinical relevance. As a result, only a reduced number of clinical trials are being conducted in which stem cells are applied.

In addition to CRT itself, stem cells can also be beneficial by providing a trophic support, by improving the survival of affected neurons (Lindvall and Kokaia 2009; Lunn et al. 2011) or acting as inflammation modulators. Not surprisingly, both epidemiological and genetic studies support an important role of neuro-inflammation in the pathophysiology of PD (Hirsch et al. 2012; More et al. 2013).

5.4 Multipotent Stem Cells

5.4.1 Human Neural Stem Cells

Human Neural Stem Cells (hNSCs) are uncommitted and multipotent cells with the ability for self-renewal that can differentiate into all the neural cells of CNS (i.e. neurons, astrocytes and oligodendrocytes). These cells can be obtained from differentiation of pluripotent stem cells and from fetal, neonatal, and adult brain.

In the human brain, NSCs are found in the developing nervous system and in two neurogenic niches of the adult brain, the subventricular zone of the lateral ventricles and the dentate gyrus in the hippocampal formation (Kempermann et al. 1997). However their regeneration potential is very limited and there is no evidence of endogenous neurogenesis in the SN in PD.

It has been shown that these cells are also present in the subventricular zone of PD patients and they are able to proliferate, which could provide a future possibility to develop novel therapeutic approaches to stimulate these cells to migrate to the striatum and differentiate into dopamine neurons (Van den Berge et al. 2011, 2013). However the number of proliferating cells is small and highly variable between

individuals and methods to expand this population of cells for clinical use remain to be established.

It is also worth noting that adult neurogenesis may be affected during the progression of PD. In fact, it has been observed that the proliferation of cells appears to be decreased in the brains of PD patients. Therefore, an alternative option may involve infusing exogenous hNSCs to replacing lost neurons (Höglinger et al. 2004).

hNSCs can be propagated *in vitro* as free-floating aggregates, called neurospheres. Neurospheres are a mixture of NSCs and progenitor cells grown in the presence of growth factors such as basic Fibroblast Growth Factor (bFGF) and Epidermal Growth Factor (EGF) (Bonnamain et al. 2012; Kallur et al. 2006). An alternative approach for the expansion of hNSCs is the genetic immortalization where cells are transduced with immortalizing genes (e.g., TERT, v-Myc or c-Myc) and supporting the proliferation by adding growth factors (Villa et al. 2009; Cacci et al. 2007).

Several types of hNSCs have been explored in order to generate midbrain DAn, including human VM neural precursors. These, in theory, should be the ideal candidates for cell therapies in PD.

Unfortunately, VM neural precursors present poor growth potential and unstable phenotypes losing their initial properties with repeated passages in culture (Villa et al. 2009; Ramos-Moreno et al. 2012). Furthermore, they survive poorly into the brain when grafted (Kim et al. 2009a), limiting them from being a stable source of human DAn.

Recently an efficient method to generate large numbers of midbrain DAn has been described. This method is based in the expansion and differentiation of neural precursor cells present in the human VM tissue by adding Wnt5a. Using this method, a sixfold increase of the number of midbrain DAn was obtained as compared to the starting VM preparation (Ribeiro et al. 2013), and could solve in part, the lack of VM tissue in future transplantation studies.

A different tactic consists of immortalizing hNSCs from the VM, although it can not be considered for clinical use. If these methodologies are successful, they could end some of the limitations described previously (Villa et al. 2009; Liste et al. 2004; Lotharius et al. 2002).

Despite all of this, an efficient method to induce midbrain dopamine neurons from NSCs in large numbers for clinical treatment is still lacking.

In a different approach, hNSCs isolated from brain biopsies have been used to treat PD patients by the company Neuro Generation Inc. In this trial, biopsied cortical and subcortical tissue samples during craniotomy were proliferated for several months and then differentiated into neurons (60 % GABAergic, 15 % DAn) and glial cells. These cells were implanted in multiple sites in the putamen. Patients showed some motor improvement, increased dopamine uptake and other clinical benefits (Lévesque et al. 2009). Further trials are being developed to determine the feasibility and efficiency of this method (see Table 5.1 and Fig. 5.1).

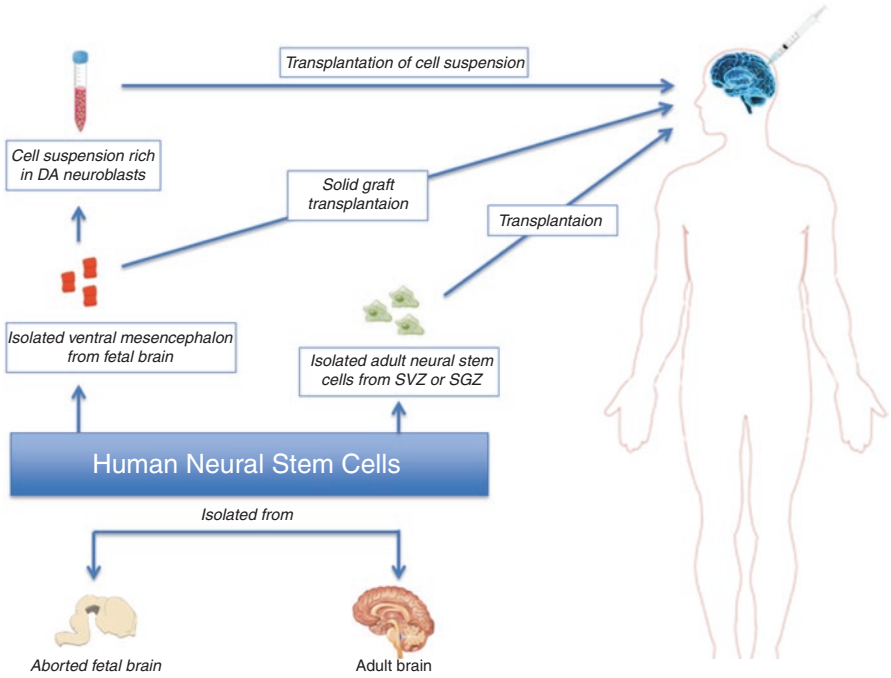

Fig. 5.1 Schematic representation of the possible use of human Neural Stem Cells for Cell Replacement Therapy (CRT) for treatment of PD

5.4.2 Human Mesenchymal Stem Cells

Human Mesenchymal Stem Cells (hMSCs), also named marrow stromal cells, may be an alternative source of multipotent stem cells that, until now, have primarily been isolated from adult bone marrow (Collins et al. 2014). However, these cells can also be found elsewhere in the body, including adipose tissue (Zuk et al. 2002), umbilical cord blood (Erices et al. 2000), dental pulp (Gronthos et al. 2000), placenta (Abumaree et al. 2013) and brain (Paul et al. 2012).

Their widespread availability throughout the body, in addition to their great proliferative potential once isolated, has made MSCs emerge in the last years as a promising approach in regenerative medicine (Teixeira and Carvalho 2013; Ryan et al. 2005).

hMSCs are stromal cells characterized by the adherence to plastic in cell culture. They exhibit positive expression of specific markers such as CD105, CD73 and CD90, and they do not express hematopoietic markers like CD34, CD45, HLA-DR, CD14, CD11B or CD19. They also show multi-lineage differentiation potential to cells of mesodermal origin (osteocytes, chondrocytes, adipocytes) (Lunn et al. 2011; Trounson and Pera 2001; Joyce et al. 2010). Recently, some evidence has shown that they can transdifferentiate towards a neural lineage

(Macias et al. 2010; Lunn et al. 2011; Satija et al. 2009; Paul et al. 2012) and even obtain a DAn phenotype (Trzaska and Rameshwar 2011; Dezawa et al. 2004; Paldino et al. 2014). Therefore, it has become plausible to use these stem cells for the generation of DA-like neurons.

In vivo studies in macaque models of PD after transplantation of dopamine producing cells induced from autologous bone-marrow derived MSCs, have proved very promising.

However, beneficial effects were not conclusively shown to be caused by dopaminergic neuron integration, but rather could be caused by neurotropic effects of the MSCs (Hayashi et al. 2013).

Another recent study using primed-fetal liver MSCs showed functional and neurochemical recovery of dopaminergic neuron activity in a 6-OHDA mouse model when transplanted directly into the striatum. However, it was also shown that not all primed MSCs differentiated into a neuron-like cell. Therefore, further studies are needed for long-term efficacy and safety before considering this cell source for CRT in humans (Kumar et al. 2016).

Furthermore, Chun et al., showed a potentially viable source of MSC-derived dopaminergic neurons differentiated from dental pulp *in vitro*, confirming morphological identity and dopamine release. The results from this study need to be taken further to verify if the cells possess the electrophysiological characteristics necessary for proper integration *in vivo* (Chun et al. 2016).

Still, the differentiation potential of hMSCs is much more limited than that of pluripotent stem cells. The trans-differentiation of MSCs from a mesodermal to a neural cell lineage is still relatively new in the field, and for this reason their use as a source for CRT in neurodegenerative disorders remains somewhat controversial. The studies that have been done show inconsistent and inconclusive results in animal models and, it is still unsure if these neuron-like cells derived from MSC can be correctly integrated into the host-neural circuitry to form synaptic connections (Joyce et al. 2010; Hardy et al. 2008; Glavaski-Joksimovic and Bohn 2013). For these reasons, more research needs to be done and better standardization procedures of MSC sources, along with improved differentiation protocols are needed to produce viable and consistent dopaminergic-like neurons before being used in clinical trials.

Currently, the discovery of the beneficial effects mediated by the hMSCs secretome (a concept defined as the proteins secreted by cells or tissues that become crucial for the regulation of subsequent cellular processes) may be a more promising approach to their use as a treatment option for PD (Teixeira and Carvalho 2013). MSCs secrete protective neurotrophic factors, growth factors and cytokines that promote protection, repair and show immunomodulatory effects. Additionally, other advantage of the use of hMSCs is that they could, in theory, circumvent the need of immunosupression in cell therapies, as they can be derived from patient own tissue. For this same reason, they are not ethically controversial when compared to hESCs, adding another reason to their growing popularity as a cell source for treatment options in several different diseases, including PD.

Paracrine factors excreted by MSCs have been shown to modulate the plasticity of host tissue, by secreting neurotrophic and growth factors such as vascular endo-

thelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF-1) (Hoch et al. 2012), brain derived neurotrophic factor (BDNF), β-nerve growth factor (β –NGF), transforming growth factor β (TGF- β), fibroblast growth factor 2 (FGF2), and glial cell derived neurotrophic factor (GDNF) which are involved in increasing neurogenesis, promoting neuronal and glial cell survival, angiogenesis, inhibition of apoptosis, immunomodulation and showing neuroprotective actions in brain (Siniscalco et al. 2010; Chamberlain et al. 2007; Teixeira and Carvalho 2013). In addition, MSCs produce extracellular matrix proteins (ECM) that could favor neural cell attachment, growth and neuritogenesis that could lead to functional neural restoration (Tate et al. 2010; Li et al. 2010).

Several studies have shown that both naïve bone marrow-derived MSCs (BM-MSCs) and neurally differentiated BM-MSCs had therapeutic effects in PD animal models. This was verified because of their capacity to regenerate and protect damaged DAn. MSCs isolated from the umbilical cord, adipose tissue and placenta have shown neuroprotective and neuro-regenerative effects in PD animal models too (McCoy et al. 2008; Mathieu et al. 2012; Park et al. 2012). Currently, MSCs isolated from adipose tissue are receiving more attention because of easier access and differentiation potential as compared to other sources of MSCs (Chang et al. 2014). In addition to the studies showing neuroprotective effects of grafted MSCs, a recent study also showed that MSCs derived specifically from adipose tissue showed a modulatory role after transplantation into the medial forebrain bundle, supporting their ability to enhance endogenous neurogenesis and adapt to a noxious microenvironment that could making them a potentially safe treatment option for PD (Schwerk et al. 2015).

Although the exact pathogenesis of PD is unknown, mounting evidence is suggesting that chronic neuronflammation is one of the causes of pathophysiology of PD. The presence of activated astrocytes and microglia leads to neurodegeneration and loss of DA neurons, making it a good treatment target by increasing cytokines such as TNFB, IL-4 and IL 10 (Wang et al. 2015). Following these findings, studies have shown that MSCs not only act through paracrine effects relying on close cell-contact to the injured area, but release soluble factors that can be involved in immunosuppression in the brain (Kim et al. 2009b) and inhibit the release of pro-inflammatory cytokines (Ng et al. 2014).

Another property of hMSCs is they are able to migrate to places of injury in animals when they are infused systemically, suggesting their migratory potential and promoting the repair process through the secretion of growth factors, cytokines, and antioxidants (Teixeira and Carvalho 2013; Vegh et al. 2013). Migration can also be induced by growth factors and chemokines released after damage that could provide migratory signals that induce activation of integrins from MSCs and up-regulation of selectins, promoting cells to interact with the endothelium, similar to leukocytes of the immune system (Martínez-Morales et al. 2013; Teo et al. 2012)

Recently, another study also confirmed the beneficial effects of combined treatment of MSC conditioned medium and neural stem cell grafting, showing behavioral and functional improvement in a PD animal model (Yao et al. 2015).

Although it is believed that all MSCs generally possess the same regenerative properties, populations isolated from different tissues are biologically heterogeneous

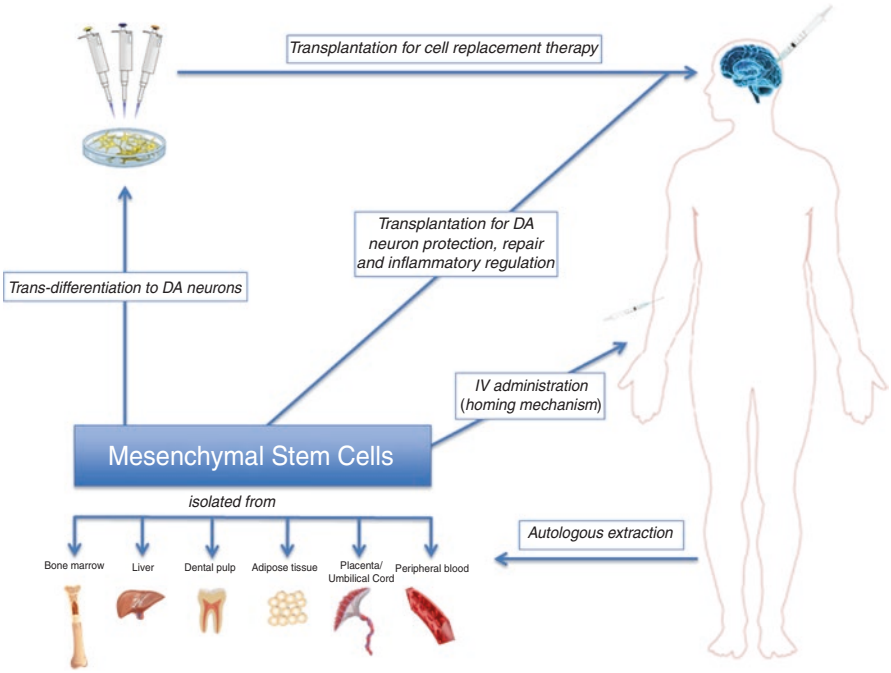

Fig. 5.2 Mesenchymal stem cells can be isolated from different tissues and infused intracerebrally or systemically for treating Parkinson´s Disease (PD) patients

and may vary in their immune-phenotype, proliferation rate and commitment to different cell lines (Paul and Anisimov 2013). Therefore, their use in clinical trials has been limited. However, an open label study from 2009 using bone marrow derived MSCs in seven patients showed immediate and short term safety to use these cells for treatment of PD, but the clinical improvement they displayed were only marginal (Venkataramana et al. 2009).

Furthermore, hMSCs from adipose tissue and bone marrow are currently being used to investigate the efficacy of autologous and allogenic treatments in PD patients with the idea of taking advantage of their immune-modulatory and trophic properties (Kitada and Dezawa 2012; Schwarz and Storch 2010]) (Table 5.1 and Fig. 5.2).

5.4.3 Human Pluripotent Stem Cells

In theory, pluripotent stem cells are the ideal material for the treatment of several diseases using cell therapy, mainly due to their ability to self-renewal and to differentiate into any cell type of the body.

However there are still several risks related to their use: the possibility of tumor formation, host immune reactions, technical questions related to correct

differentiation with the desired phenotype and ethical issues (in the case of human embryonic stem cells). Currently, the use of hPSCs is extremely regulated in most countries, leading to a reduced number of approved clinical trials involving their use.

5.4.4 Human Embryonic Stem Cells

The first human Embryonic Stem Cells (hESCs) were isolated from the inner cell mass of the blastocyst, in 1998 (Thomson et al. 1998). These cells are characterized by their self-renewal capacity and the potential to differentiate towards specialized cells of all germ layers (endoderm, ectoderm and mesoderm). Due to these properties, hESCs can be a suitable cell source for cell-based therapies.

The clinical application of hESCs in neurodegenerative diseases such as PD depends on their efficient differentiation into the DAn phenotype. In this regard, functional VM DAn have been obtained using different protocols. Currently, the most effective ones are those based in dual SMAD inhibition (Grealish et al. 2014; Kirkeby et al. 2012, 2013) and conversion of hESCs into precursors of floor plate that, after exposure to agonists of Shh and Wnt signaling pathways, are efficiently converted to DAn (Kriks et al. 2011; Chambers et al. 2009). In both cases, neurons generated can survive and integrate into the lesioned brain with long-term functional benefits, encouraging the research aimed at using hESCs for treating PD.

The recent work of Malin Parmar's group has demonstrated the DAn derived from hESCs can survive and innervate the brain regions of interest after grafting into the brain of parkinsonian rats (Grealish et al. 2014). Demonstrating that cell transplantation of hESC-derived DAn are functionally comparable to that of neurons derived from fetal tissue.

However, the use of hESCs is still associated with several problems including ethical issues, phenotype instability, controlling cell proliferation and correct differentiation and maturation into the desired phenotype (Petit et al. 2014; Cho et al. 2008; Martínez-Morales et al. 2013).

Furthermore to optimize the use of hESCs in clinical application, it is essential to avoid any risk of contamination, including xenogenic contamination (from cell culture reagents or feeder cells). This implies that neuronal precursors and neurons differentiated from hESCs must be obtained in GLP/GMP (Good Laboratory and Manufacture Procedures) conditions from the blastocyst isolation.

Besides, the work with hESCs is being extensively regulated in most countries. The guidelines ranging from controlled permissiveness to absolute prohibition (Condic and Rao 2010).

Finally, another challenge is the possibility of graft rejection. However, this problem could be avoided by the creation of banks of cells from immunologically diverse donor cells to include a high diversity of HLA types. Due to these limitations no clinical trial has been conducted with these cells in PD yet. But maybe not for long, as there are already planned at least two clinical trials with hESCs, one in Europe and another in the USA, to begin in the next few years (Abbott 2014).

5.4.5 Human Parthenogenetic Embryonic Stem Cells

The parthenogenetic cells are pluripotent stem cells obtained from unfertilized oocytes by the suppression of the second meiotic division, generating diploid cells carrying only the maternal chromosomes (Barker et al. 2016; Revazova et al. 2007). These cells are named parthenogenetic human embryonic stem cells (phESCs), show similar morphology to hESCs, express the typical pluripotency markers and show high levels of activity for telomerase and alkaline phosphatase (Revazova et al. 2007). They also generate embryoid bodies *in vitro* and teratoma formation after infusion in immunodeficient mice (Revazova et al. 2007).

Parthenogenetic human embryonic stem cells could be a good alternative to hESCs derived by somatic nuclear transfer (SCNT) since the process of parthenogenesis is relatively simple as compared to SCNT and does not require complex equipment (Gonzalez et al. 2015b; Revazova et al. 2007). These cells are not fertilized or activated via sperm entry and they can circumvent the ethical issues related to the use of hESCs.

However the lack of one parental contribution makes them different from hESC or hiPSCs. For this reason, their clinical use could be problematic, as it could affect the cell cycle and their ability to differentiate properly.

The first clinical trial using human pluripotent stem cells to treat PD was approved at the end of 2015 by the Australian government. The study will be carried out at the Royal Melbourne Hospital in Melbourne, Australia by the company International Stem Cell Corporation (ISCO). This is a Phase 1/2 trial in twelve patients with PD. For transplantation, the company is planning to use a population of NSCs previously generated from the parthenogenetic pluripotent stem cells (Barker et al. 2016).

5.4.6 Human Induced Pluripotent Stem Cells

The first ES-like cells from adult somatic cells were generated by Takahashi and Yamanaka through the overexpression of a few transcription factors (Takahashi and Yamanaka 2006). These ES-like cells were generated by transducing mouse embryonic fibroblasts (MEFs) with retroviruses that expressed Oct3/4, Sox2, Klf4, and c-Myc (abbreviated as OSKM). The combination of the four transcription factors gave rise to the known "induced pluripotent stem cells (iPSCs)" (Takahashi and Yamanaka 2006).

A year later it was reported the generation of human iPSCs from fibroblasts by two different laboratories, Yamanaka's team (Takahashi et al. 2007) and Thomson' s group (Yu et al. 2007). The first one used OSKM factors, while the second included NANOG and LIN28.

Human iPSCs are similar to hESCs in many aspects like morphology, expression of pluripotency factors, epigenetic marks, differentiation potential *in vivo* and *in*

vitro and the ability to generate viable chimeras (Martínez-Morales and Liste 2012; Phanstiel et al. 2011).

Certainly the cell reprogramming technology and the ability to generate iPS cells has been a breakthrough in the field of biomedical and clinical research. Since these cells can be used as *in vitro* models of diseases and for autologous grafting (no immunosuppression should be necessary). Further, they do not generate ethical controversies as can be derived from adult tissues.

Focusing in PD, human DA precursors have been efficiently derived from hiPSCs isolated from control, or from PD patients by using similar protocols to those used for differentiation of hESCs (Kriks et al. 2011; Soldner et al. 2009; Hargus et al. 2010; Nguyen et al. 2011; Sánchez-Danés et al. 2012; Kirkeby et al. 2012). When transplanted into the brain of lesioned rats, hiPSCs were able to survive and differentiate into DAn, showing a significant improvement in the motor tests, without generating tumors (Doi et al., 2014).

However we must be careful, since iPS cells have, at least, the same challenges and risks as hES cells; in addition to the problems associated with the reprogramming process itself and that they can be derived from the tissue of patients (they will carry the same genetic defects as the patient cells).

In fact, most efficient strategies for hiPSCs generation are based in the use of retroviral and lentiviral vectors that can integrate into the somatic cell genome, increasing the risk of oncogenic transformation and/or insertional mutagenesis. Currently, to avoid these risks, important advances have been made in the field by using excisable vectors (Soldner et al. 2009), non-integrative vectors (Stadtfeld et al. 2008), the use of direct protein or mRNA delivery (Bernal 2013; Zhou et al. 2009) and the addition of different chemical compounds (Masuda et al. 2013).

Some studies have identified genomic instability, as well as epigenetic and genetic abnormalities associated with the reprogramming process itself (which could be expected because most of the reprogramming factors used possess oncogenic potential (Revilla et al. 2015; Pasi et al. 2011; Lister et al. 2011). Another obstacle to overcome in hiPS-based cell technology before its therapeutic application is the risk of tumor formation. This risk is associated in part with the existence of proliferating cells after implantation but also with the phenotype heterogeneity of the differentiated cells as it happens in hESCs (Fu and Xu 2012; Thomson et al. 1998; Ben-David and Benvenisty 2011).

In any case, the expectations placed on hiPSCs are huge and several clinical trials are already planned with these cells for PD waiting government approval. One is expected to start soon in Japan led by the group of Jun Takahashi (Morizone and Takahashi 2016; Drouin-Ouellet and Barker 2014; Garber 2013) (Fig. 5.3).

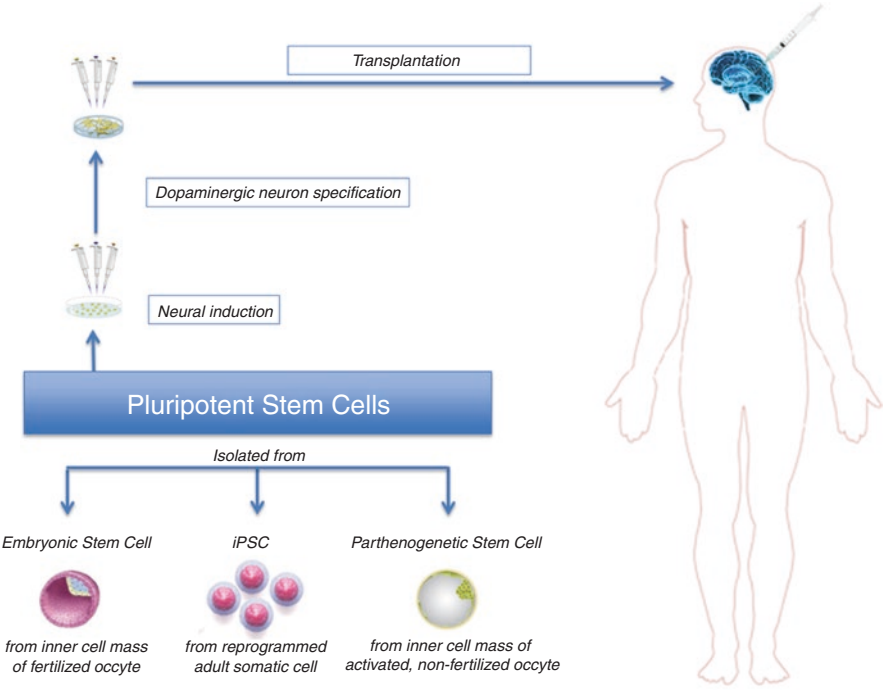

Fig. 5.3 Different types of human pluripotent stem cells available for CRT in PD after differentiation into Dopaminergic neurons

5.5 Conclusions and Future Directions

Conventional pharmacological treatments for most neurological disorders, including PD, only provide some symptomatic improvement but do not stop the progression of the degeneration. Therefore there is a clear need for alternative therapeutic strategies. The development of cell-replacement therapies using stem cells can provide substantial benefits for PD patients, as shown after transplantation of fetal cellular suspensions of dopaminergic precursors.

However, clinical studies to date have shown that there are still many gaps in terms of safety, effectiveness and overall methodology used in these implants. Among the aspects that need improvement, would be: (i) the development and standardization of surgical methods for cell transplantation and biological assays to evaluate the survival and effectiveness of the grafts. (ii) A good understanding of the effects of inflammatory and immunological processes on the progression of PD and on the implanted neurons. (iii) A better design and planning of clinical trials in terms of patient selection, evaluation criteria and patient follow-up. (iv) Identification of the best type of cell to be used as a source of DAn and standardization of protocols for the optimal production of these cells.

In relation to this last point there has been enormous progress in recent years; especially with regard to the generation of fully functional DAn from hESCs and hiPSCs. This has been possible thanks to a better knowledge of the signaling molecules that regulate embryonic development of DAn.

The DAn derived from human pluripotent stem cells can be obtained in large quantities in culture and have been shown to be able to survive long term implantation in preclinical models of PD being functional in a similar way to that observed with human fetal VM neurons. The main advantage of hiPSCs over hESCs is that they can be obtained from somatic cells of the own patient, therefore, could prevent immune rejection.

The derivation of clinically safe hiPSCs and their subsequent differentiation into DAn *in vitro* could provide excellent tools to replace the degenerated neurons in PD patients.

Furthermore the increasing results obtained from experiments using MSCs is also beginning to provide potential treatment options for PD. Though more studies need to be completed to confirm the safety and efficacy of the use of trans-differentiated MSCs for CRT, significant strides have been made in the use of MSCs as a neuroprotective treatment option. These cells have been show to promote neural and glial cell survival, control angiogenesis, inhibit apoptosis, have immunomodulatory functions and show neuroprotective actions through the release of a number of different neurotrophic and survival-promoting growth factors. A few clinical trials are already underway, utilizing allogenic and autologous-derived stem cells intravenously administered to see the beneficial effects of MSCs in humans.

In short, the perspective of an unlimited source of cells, in combination with the promising preclinical results suggests that CRT technology can be very close to a realistic clinical application in PD.

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Chapter 6
Mesenchymal Stromal Cell Therapy for Neonatal Hypoxic-Ischemic Encephalopathy

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6.1 Introduction

It has been estimated that intrapartum hypoxia-ischemia/asphyxia was responsible for 1.15 million new cases of neonatal encephalopathy (NE) in 2010, resulting in a total of 413,000 children with neurodevelopmental impairments worldwide. Impressively, more than 85 % of these new cases occurred in South–Southeast Asia and sub-Saharan Africa (Lee et al. 2013). More recently, the Global Burden of Disease 2013 Study reported that NE following birth trauma and asphyxia was responsible for more than 10 % (643,765 deaths) of the 6.28 million deaths that occurred in children under 5 years of age in 2013, representing the third most common cause of death in this age group (Kyu et al. 2016). As suggested by Volpe (Volpe 2012), these cases should be referred to as neonatal hypoxic-ischemic encephalopathy (HIE), in order to differentiate them from cases in which NE occurred due to other causes.

Although therapeutic hypothermia has been implemented as the standard treatment for patients with moderate/severe HIE, the number needed to treat was estimated to be 6–8 (Davidson et al. 2015; Tagin et al. 2012; Papile et al. 2014). This

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means that a large number of children would still benefit from additional neuroprotective or neurorestorative strategies.

There has been a growing interest in the development of cell-based therapies for HIE. A special emphasis has been given to the study of mesenchymal stromal cells (MSC), which display a number of characteristics that make them very attractive for therapeutic applications in HIE (Pimentel-Coelho and Mendez-Otero 2010). This chapter discusses current preclinical evidence on the mechanisms by which MSC could contribute to brain repair in HIE.

6.2 Mesenchymal Stromal Cells

MSC are a heterogeneous self-renewing cell population found in the stroma of several tissues (Bianco and Gehron Robey 2000). *In vitro*, they have a spindle-shaped morphology, form single cell-derived colonies, are plastic-adherent, and proliferate in the presence of fetal calf serum (Conget and Minguell 1999; Pittenger et al. 1999; Friedenstein et al. 1976). Moreover, MSC have the potential to differentiate into adipocytes, chondroblasts, and osteoblasts (i.e., cells of the mesodermal lineage) (da Silva Meirelles et al. 2006).

MSC can be obtained from a variety of adult tissues, including bone marrow, adipose tissue and peripheral blood, among others (da Silva Meirelles et al. 2006). For instance, MSC account for less than 0.01 % of the nucleated cells in the bone marrow (Pittenger et al. 1999) and this is the most studied and well-characterized source of MSC in adults. However, the isolation of bone marrow-derived MSC (BM-MSC) requires bone marrow aspiration, a minimally invasive procedure (Malgieri et al. 2010). An alternative source that has been widely investigated is the adipose tissue, which contains large numbers of MSC (up to 3 % of all cells) that can be easily obtained by minimally invasive procedures such as liposuction aspiration and needle biopsies (Baer and Geiger 2012). Although aging can have a negative impact on the function of adipose-tissue derived MSC (AT-MSC) and BM-MSC (Choudhery et al. 2014; Zhou et al. 2008), it is not clear how the ageing process affects their therapeutic potential.

MSC can also be isolated from neonatal tissues, including the umbilical cord and placenta. The human umbilical cord Wharton's jelly is a rich, reliable and easily accessible source of MSC (hUC-MSC) that display a high proliferative potential (Zhang et al. 2014). The human umbilical cord blood is also a source of MSC (hUCB-MSC) that display high proliferative rates, although there are critical factors that can limit their isolation, such as the time from collection to processing (Bieback et al. 2004; Zhang et al. 2011).

There are increasing evidence that MSC can promote regenerative processes and induce brain repair in a wide variety of preclinical models of neurological diseases (Rosado-de-Castro et al. 2013; Mesentier-Louro et al. 2016), including the Rice-Vannucci rodent model of HIE (Pimentel-Coelho et al. 2010; Vannucci and Vannucci 2005) and the ovine model of global hypoxia-ischemia in the developing brain (Jellema et al. 2013).

6.3 The Therapeutic Efficacy of Mesenchymal Stromal Cells in Preclinical Animal Models of Neonatal Hypoxia-Ischemia

6.3.1 Mesenchymal Stromal Cells from Adult Tissues

Van Velthoven and colleagues have shown that intracerebrally transplanted BM-MSC improved the behavioral outcome and decreased the lesion size in a rodent model of HIE (van Velthoven et al. 2010a, c). Beneficial results were also obtained when BM-MSC were intravenously (Yasuhara et al. 2008), intracardially (Lee et al. 2010), subcutaneously (Cameron et al. 2015) and intranasally transplanted (van Velthoven et al. 2010b; Donega et al. 2013; Donega et al. 2014b).

Furthermore, it has been demonstrated that the intracerebroventricular administration of AT-MSC recovered the locomotor activity and improved the cognitive function in a model combining cerebral hypoxia-ischemia and systemic inflammation (induced by the intraperitoneal injection of lipopolysaccharide) in rats. AT-MSC attenuated brain inflammation and restored the expression of several growth and neurotrophic factors in the brain (Park et al. 2013).

6.3.2 Mesenchymal Stromal Cells from Neonatal Tissues

The intracerebral transplantation of hUC-MSC was shown to promote the restoration of learning and memory functions via the secretion of interleukin (IL)-8, which enhanced angiogenesis in the hippocampus via the JNK signaling pathway (Zhou et al. 2015). Moreover, the administration of hUC-MSC via intravenous or intraperitoneal routes improved the long-term functional outcome of rats (Zhang et al. 2014; Zhu et al. 2014).

Finally, there is evidence that intracerebrally transplanted hUCB-MSC and rat placenta-derived MSC can contribute to the functional recovery of hypoxic-ischemic rats (Xia et al. 2010; Ding et al. 2015a, b).

6.4 Mechanisms of Action of Mesenchymal Stromal Cell-Based Therapies in Animal Models of Neonatal Hypoxia-Ischemia

It is already well established that MSC do not transdifferentiate into functional neural cells *in vivo* (Abraham and Verfaillie 2012; Lin et al. 2015). Instead of replacing lost cells, MSC seem to exert their therapeutic effects through paracrine and contact-dependent signaling.

Zhou and coworkers (Zhou et al. 2015), for example, identified IL-8 as key player in the paracrine action of human umbilical cord-derived MSC in a rodent model of HIE. Silencing of IL-8 gene expression in MSC abolished the beneficial cognitive effects of a single intracerebroventricular injection of MSC. In addition, another study from the same group showed that rat BM-MSC exerted their therapeutic effects on cognition through the secretion of IL-6 and that this cytokine was involved in the protection of astrocytes in an *in vitro* model of oxygen/glucose deprivation (Gu et al. 2016).

The paracrine action of MSC is also supported by studies on the composition and biological activity of the conditioned medium of cultured MSC. IL-6, IL-8, vascular endothelial growth factor (VEGF) and the chemokine CCL2 are some of the factors most commonly found in the secretome of non-stimulated MSC (Ranganath et al. 2012). Moreover, the secretome can be modified by genetic manipulation (van Velthoven et al. 2014) or by changes in culture conditions (Ranganath et al. 2012). For instance, a recent study showed that the intranasal delivery of MSC genetically engineered to secrete brain-derived neurotrophic factor (BDNF) improved the motor function, decreased lesion volume and induced cell proliferation in the ischemic hemisphere, whereas the treatment with MSC modified to secrete epidermal growth factor-like 7 (EGFL7) only improved the motor function after HIE (van Velthoven et al. 2014).

The neuroprotective action of MSC-released factors was further demonstrated by Wei et al. (Wei et al. 2009), who treated neonatal rats with an intravenous injection of the conditioned medium of AT-MSC either 1 h before or 24 h after the hypoxic-ischemic insult. Both treatment protocols were effective in reducing brain tissue loss and preventing the development of long-term spatial learning deficits. Insulin-like growth factor-1 (IGF-1) and BDNF were partially responsible for the neuroprotective effects of the prophylactic administration of conditioned medium. These findings were corroborated by *in vitro* experiments showing that the conditioned medium protected cerebellar granular neurons against serum and K^+ deprivation-induced cell death, as well as against glutamate excitotoxicity.

Taken together, these studies indicate that it may not be possible to identify a single factor that could explain the multiple actions of MSC. Future studies are necessary to compare the efficacy of MSC versus MSC-conditioned medium in animal models of HIE. A possible advantage of the cell therapy is the fact that MSC adapt their secretome in response to changes in the environment. For instance, the co-culture of MSC with brain extracts from hypoxic-ischemic animals increased the mRNA expression of BDNF and nerve growth factor (NGF) by MSC (Donega et al. 2014b). Therefore, MSC could act as a "site-regulated drug-store", as suggested by Caplan and Correa (Caplan and Correa 2011). On the other hand, the administration of cells is not devoid of potential risks (Boltze et al. 2015) and the composition of the conditioned medium can be modulated by different types of stimuli.

6.4.1 *Immunomodulatory and Neuroprotective Actions of Mesenchymal Stromal Cells*

MSC display potent immunosuppressive and immunoregulatory activities, by interacting with cells of the innate and adaptive immune system. This led to the approval of Prochymal® (remestemcel-L) as the first MSC-based product for the treatment of pediatric graft versus host disease in Canada and New Zealand (Zhao et al. 2016).

With regard to the central nervous system, MSC have been shown to modulate the phenotype and function of microglia. For instance, MSC can attenuate the release of pro-inflammatory factors by lipopolysaccharide-stimulated primary cultured newborn microglia and microglial cells lines *in vitro* (Hegyi et al. 2014; Liu et al. 2014; Jose et al. 2014). These effects seem to be mediated by the secretion of soluble factors by MSC, since they can be reproduced by the treatment of microglial cells with MSC-conditioned medium (Ooi et al. 2015). Although it is unlikely that a single MSC-derived factor could be responsible for this anti-inflammatory action, it has been demonstrated that tumor necrosis factor (TNF)-α stimulated gene/protein 6 (TSG-6) (Liu et al. 2014) and the chemokine CX3CL1 (Giunti et al. 2012) were at least partially implicated in the modulation of microglial activity by MSC.

Several studies have shown that MSC can reduce the recruitment or proliferation of microglial cells/macrophages in rodent models of HIE and neonatal stroke (Kim et al. 2012; Zhu et al. 2014; Donega et al. 2014a, b; van Velthoven et al. 2010a). Similar findings were reported by Jellema and colleagues (Jellema et al. 2013), who used an ovine model of global hypoxia-ischemia in the preterm brain to evaluate the anti-inflammatory effects of a single intravenous administration of human BM-MSC. They showed that the treatment was able to reduce the number of microglial cells and CD3-positive T lymphocytes in the injured subcortical white matter. Moreover, MSC suppressed the proliferation of CD4-positive helper T cells in the spleen of sham-operated and hypoxic-ischemic animals. These immunomodulatory effects were accompanied by morphological and functional improvements, such as the decrease of white matter injury, brain atrophy and electrographic seizure burden.

A potential confounding factor in the interpretation of these results is the fact that, in most of these studies, MSC treatment also decreased the number of dying neurons. Regardless of the administration route, MSC were shown to decrease the loss of cerebral white and gray matter in models of HIE (Donega et al. 2013, 2014a; van Velthoven et al. 2010b, c). It is known that the microglial response is directly related to the degree of neuronal loss. Therefore, it is difficult to conclude whether the decrease in the number of microglial cells *in vivo* is a direct consequence of MSC immunomodulatory activity or an indirect effect related to the neuroprotective action of MSC.

In addition, it may not be correct to conclude that the neuroprotective effect of MSC is secondary to its effects on microglia. *In vitro* studies support the notion that MSC or MSC-conditioned medium can directly protect hippocampal and cortical

neurons from oxygen/glucose deprivation, in the absence of microglial cells (Scheibe et al. 2012; Piscioneri et al. 2015). Therefore, further studies are necessary to determine whether MSC can potentiate the recently described protective effects of microglia in the neonatal brain (Faustino et al. 2011; Fernandez-Lopez et al. 2016), while suppressing the toxic effects related to prolonged microglial activation (Saijo and Glass 2011).

Besides the local anti-inflammatory effect, human MSC can attenuate systemic inflammation. For instance, a recent study found that the intracerebral administration of placenta-derived MSC decreased the plasma levels of TNF-α, IL-17, interferon (IFN)-γ and increased the levels of the anti-inflammatory cytokine IL-10 in a rat model of HIE. In addition, the treatment increased the number of regulatory T cells (Tregs) in the spleen (Ding et al. 2015a).

6.4.2 Mesenchymal Stromal Cells and Neuroplasticity

There is strong evidence that MSC can induce neurogenesis and oligodendrogenesis in the endogenous stem cell niches. The intracerebral administration of BM-MSC 3 days after hypoxia-ischemia in mice promoted cell proliferation and increased the number of newly born neurons and recently divided oligodendrocyte progenitors in the hippocampus and cerebral cortex, whereas the number of newly formed microglia was reduced in both areas (van Velthoven et al. 2010a, c). Mice treated with two doses of BM-MSC (at 3 and 10 days after HIE) exhibited a better functional recovery, in comparison to animals that received a single dose. Moreover, a second dose of BM-MSC at 10 days restored the connection between the injured ipsilateral motor cortex and the contralateral spinal cord, promoting axonal remodeling of the motor tracts. This effect was accompanied by an increase in neuritogenesis and synaptogenesis, as assessed by GAP43 and synaptophysin staining, respectively (van Velthoven et al. 2010c)

The intranasal administration of BM-MSC can stimulate neurogenesis in the subventricular zone and at the lesion site, as demonstrated by the increased number of neuroblasts and neural precursor cells at both regions after the therapy (Donega et al. 2014b). Intranasally injected BM-MSC also reduced the activation of astrocytes, therefore reducing the glial scar, which is known to inhibit axon regeneration, even though it acts as a barrier that prevents the spread of tissue damage (Donega et al. 2014a, b).

The main limitations of the endogenous regenerative response of the subventricular zone in models of HIE are the poor survival of newborn neurons and the preferential differentiation of neuroblasts into calretinin-positive striatal interneurons (Yang et al. 2007, 2008). However, a recent study has shown that the delayed treatment (7 days after hypoxia-ischemia) with a subcutaneous injection of BM-MSC was able to increase the number of newly-generated DARPP-32-positive striatal medium-spiny projection neurons in the striatum 1 week after the treatment, particularly in the group that received a higher dose (750,000–1,000,000 cells) (Cameron et al. 2015).

6.5 Routes of Administration, Cell Doses and Therapeutic Time-Window

MSC have a strong tropism for inflamed and injured sites, such as tumors and ischemic lesions in the brain (Sasportas et al. 2009; Kim et al. 2008). Regarding the developing brain, it has been demonstrated that MSC can survive for several weeks after the intracerebral or intracerebroventricular transplantation in neonatal animals (Phinney et al. 2006; Croitoru-Lamoury et al. 2006; Chen et al. 2010). In an interesting study, Chen et al. (Chen et al. 2010) used magnetic resonance imaging (MRI) for the in vivo tracking of intracerebrally injected micrometer-sized paramagnetic iron oxide particles (MPIO)-labeled MSC in a model of excitotoxic injury to the immature brain. They found that, while the cells remained at the injection site in control animals, there was an extensive migration of BM-MSC to the lesioned sites in the contralateral hemisphere in 2 out of 4 rats. In 3 of these animals, the cells also migrated caudally along the fornix and fimbria of hippocampus.

Several routes have been used for the administration of MSC in models of HIE, including the intracerebral (Xia et al. 2010; van Velthoven et al. 2011; 2010c), intracerebroventricular (Park et al. 2013, 2015; Gu et al. 2016), intravenous (Zhang et al. 2014; Jellema et al. 2013), intraperitoneal (Zhang et al. 2014; Zhu et al. 2014), subcutaneous (Cameron et al. 2015), intra-cardiac (Lee et al. 2010) and intranasal routes (Donega et al. 2013, 2014a, b, 2015). The intra-arterial route has not been tested, but it should be noted that this route was associated with important side effects in adult animals, including the formation of micro-occlusions and stroke (Cui et al. 2015; Janowski et al. 2013).

In comparison to the intracerebroventricular and intracerebral routes, only a relatively small number of donor cells could be found in the brain after the systemic injection of rodent or human MSC in models of HIE. Moreover, two studies showed that the intravenous route was more effective in delivering MSC to the hypoxic-ischemic brain than the intraperitoneal one (Ohshima et al. 2015; Zhang et al. 2014). In spite of that, rats treated with hUC-MSC either through the peritoneal cavity or the jugular vein exhibited a better spatial learning performance after HIE, with virtually no differences between the routes (Zhang et al. 2014).

A limitation of the intravenous route is the entrapment of MSC in the lungs (Ohshima et al. 2015; Fischer et al. 2009), although this may be a necessary step for the activation of MSC in some situations, such as in the model of acute myocardial infarct (Lee et al. 2009).

The intranasal route has been extensively studied by Donega and colleagues (Donega et al. 2013, 2014a, b, 2015). They have demonstrated that the intranasal administration of mouse MSC is safe (Donega et al. 2015) and improves several functional and structural outcomes in hypoxic-ischemic mice (Donega et al. 2013). A dose-escalation study showed that 0.5×10^6 cells was the minimal effective dose, although better results were obtained with the intranasal instillation of 1×10^6 cells. This study also showed that MSC have a broad therapeutic time-window of at least 10 days and that there were no beneficial effects when the treatment was delayed to

14 days after the insult. Interestingly, MSC migrated from the nostrils to the lesion sites only when injected up to 10 days (but not at 14 days) post-insult, suggesting that the treatment efficacy depended on the migration of MSC to the brain (Donega et al. 2013). In a subsequent study they observed that MSC could be found near the cortical lesions as early as 2 h after administration, indicating that MSC may have alternative routes to reach the cerebral cortex, besides the migration through the brain parenchyma (Donega et al. 2014b). Furthermore, it was suggested that the chemokine CXCL10 is probably involved in the recruitment of human MSC to the brain after the intranasal injection (Donega et al. 2014a).

In conclusion, additional translational studies are needed to optimize MSC-based therapies for HIE, by the identification of the best route of administration, cell dose requirements and the therapeutic time window.

6.6 Future Directions

6.6.1 Understanding the Role of Mesenchymal Stromal Cell-Derived Extracellular Vesicles

The beneficial effects of transplanted MSC can be attributed to their ability to secret paracrine factors and modulate the local environment and the local and systemic immune responses. Recently, it has been reported that MSC secrete extracellular vesicles, including microvesicles and exosomes (Lai et al. 2010). These lipid vesicles contain proteins, mRNA, microRNAs and lipid mediators able to produce an immediate response in target cells (Valadi et al. 2007; Record et al. 2011; Subra et al. 2010).

It has been reported that MSC-derived exosomes (Exo-MSC) can transfer the microRNA 133b (mIR-133b) into neurons (Xin et al. 2012). A subsequent study from the same group showed that the intravenous administration of Exo-MSC resulted in the induction of neurogenesis, angiogenesis, and functional recovery in a model of middle cerebral artery occlusion in adult animals (Xin et al. 2013). Furthermore, the intravenous administration of Exo-MSC was shown to improve cognitive and sensorimotor functional recovery, promote neurogenesis, increase the number of endothelial cells in the lesion boundary zone and decrease brain inflammation after traumatic brain injury in adult rats (Zhang et al. 2015).

At present, the therapeutic potential of extracellular vesicles in experimental models of HIE has not been assessed and the possible mechanisms that are involved in the reparative/pro-regenerative actions of Exo-MSC in the damaged central nervous system remain to be determined. Proteomic studies have shown that human Exo-MSC contain neurotrophin 5 (NT5) and BDNF, which are known to promote neuronal survival (Lai et al. 2012), but further studies are needed to identify the

specific molecular constituents of Exo-MSC (microRNAs, mRNAs or proteins) that are required for their therapeutic effects.

6.6.2 Combining Cell-Based Therapies with Hypothermia

Therapeutic hypothermia is the only approved treatment for HIE (Davidson et al. 2015). Clinical trials aimed to investigate the safety and efficacy of MSC-based therapies for HIE will have to test the new intervention in newborns who have been, are being or will be cooled. Preclinical studies should, therefore, determine the possible interactions between the two treatments.

We found only one study in which the intracerebroventricular injection of hUCB-MSC preceded the initiation of hypothermia in hypoxic-ischemic rats. A synergistic therapeutic effect was observed in several outcomes, including infarct volume, cell death in the penumbra and recovery of the sensorimotor function. Additionally, the combined therapy was more effective in reducing brain inflammation, as demonstrated by a decrease in the number of macrophages/microglia in the penumbra and a reduced expression of pro-inflammatory cytokines in the cerebrospinal fluid (Park et al. 2015).

6.7 Conclusion

Preclinical studies have indicated that transplanting MSC from diverse sources lead to neuroprotection in animal models of HIE (Fig. 6.1). Even though the exact molecular mechanisms behind these effects are not fully known, they appear to be mediated by paracrine factors capable of inducing immunomodulation, neurogenesis, angiogenesis and neuroplasticity. Further studies are necessary to improve the understanding of the mechanisms of MSC transplantation, as well as the optimal cell sources, doses and time window for transplantation. Also, more research must be carried out to evaluate the possibility of combining MSC therapy with therapeutic hypothermia and the potential of translating preclinical findings to clinical trials.

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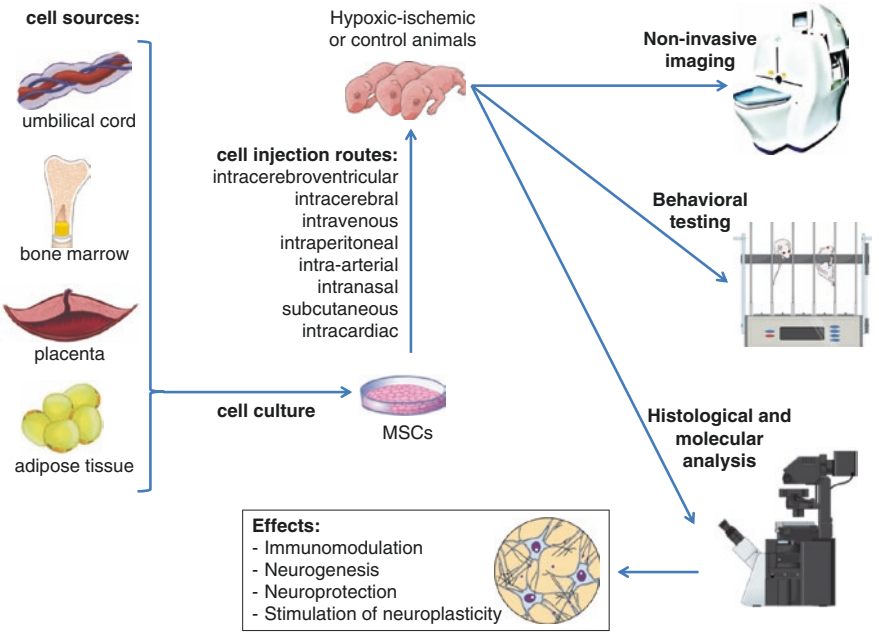

Fig. 6.1 Schematic illustration of cell sources, injection routes and observed effects of mesenchymal stromal cell (MSC)-based therapies in preclinical models of neonatal hypoxic-ischemic encephalopathy

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Chapter 7
Stem Cell Therapy for Autism

Phuc Van Pham

Abbreviations

ALS	Amyotrophic lateral sclerosis
AS	Ankylosing spondylitis
ASDs	Autism spectrum disorders
BMMNCs	Bone marrow-derived mononuclear cells
BDNF	Brain-derived neurotrophic factor
CARS	Childhood Autism Rating Scale
DCs	Dendritic cells
HSCs	Hematopoietic stem cells
iPSCs	Induced pluripotent stem cells
MSCs	Mesenchymal stem cells
MSA	Multiple system atrophy
NK	Natural killer cells
Treg cells	Regulatory cells
SS	Sjögren's syndrome
SLE	Systemic lupus erythematosus
EBMT	The European Bone Marrow Transplantation
UCMSC	Umbilical cord derived mesenchymal stem cells

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7.1 Introduction

Autism spectrum disorders (ASDs) are complex neuro-developmental disorders. ASDs result in dysfunction in social interaction and communication skills, and also lead to restricted interests and repetitive stereotypic verbal and non-verbal behaviors, which impact the ability of the patient to relate to others. Although ASD patients present with various abnormalities, the three main ones which are considered to be core symptoms of ASDs are cognitive, emotional and neurobehavioral abnormalities.

The prevalence of these disorders has dramatically increased in recent years in the United States and other countries. Although ASDs do not cause death they are, nonetheless, considered to be serious diseases which impact the quality of life of the patient. Children with ASDs, for instance, require special and intensive parental, school, and social support.

Decade-long studies from 2005 to now have shown that there are increasing more abnormalities seen in children with autism. A broad range of biochemical, toxicological and immune processes are impacted in ASD patients. These include oxidative stress, endoplasmic reticulum stress, decreased methylation capacity, limited production of glutathione, mitochondrial dysfunction, intestinal dysbiosis, increased toxic metal burden, and immune dysregulation (including autoimmunity). In recent years, several gene mutations have been found in ASD patients; however, these mutations have been inconsistent and therefore still controversial.

For a long time, ASDs were not considered as diseases. Indeed, ASDs were mostly considered as social or emotional disorders in children. These disorders were thought to gradually improve and disappear in adulthood. Due to this reason, few treatments were even suggested for ASD children. Almost all "treatments" were behavioral, nutritional, and educational approaches.

In this chapter, we summarize and update the latest research results on ASD pathophysiology and the use and efficacy of stem cells for the treatment of ASDs.

7.2 ASD: Immune Diseases

ASDs are associated with abnormality of the nervous system development during growth of the fetus. The abnormalities observed include an increase in the number of neurons (Courchesne et al. 2011), increase in neuronal dendritic volume and synapses (Hutsler and Zhang 2010), and increase in the number and size of microglial cells (Morgan et al. 2012). Some mutations of other synaptic cell adhesion molecules have also been discovered (Bourgeron 2009; Ebert and Greenberg 2013).

The correlation between the immune system and ASDs have been studied for a long time. The first report to show a relationship between the immune system and ASDs was represented by Money et al. (1971). These investigators demonstrated an association between family history of immune system dysfunction and ASDs. Since

that time, an increasing number of studies on the link of the immune system and ASDs have been performed (Atladottir et al. 2009; Enstrom et al. 2009; Torres et al. 2002, 2006, 2012).

To date, three major observations have supported the relation of ASDs to the immune system:

– Firstly, there was an association of family history of autoimmune diseases and ASD (Atladottir et al. 2009); in this study, the authors showed that the risk of ASDs increased in children with a maternal history of rheumatoid arthritis, celiac disease, and type 1 diabetes (Atladottir et al. 2009).
– Secondly, several immunological biomarkers have been noted in the autistic populations, particularly auto-antibodies (Connolly et al. 1999, 2006; Singh et al. 1993, 1998; Todd et al. 1988).
– Thirdly, immunogenetic studies have shown that human leukocyte antigen is associated to some genes related to ASDs (Al-Hakbany et al. 2014; Gough and Simmonds 2007; Liu et al. 2003; Puangpetch et al. 2015).

7.3 Stem Cell Therapy for Autism

7.3.1 Preclinical Trials

In a recent study, Segal-Gavish et al. (2016) showed that MSC transplantation could help treat the symptoms of autism in mouse models. In their study, the authors used BTBR mice for the autism model as these mice have decreased brain-derived neurotrophic factor (BDNF) signaling and reduced hippocampal neurogenesis (Scattoni et al. 2013). After MSC transplantation, treated mice exhibited a reduction of stereotypical behaviors, a decrease in cognitive rigidity and an improvement in social behavior. Tissue analysis also supported these observations; an increase of BDNF protein levels was observed in the hippocampus (Segal-Gavish et al. 2016).

7.3.2 Clinical Trials

There have been discussions about stem cell therapy for autism since 2007 (Ichim et al. 2007). The first clinical trial of stem cells for autism was reported in 2013 (Sharma et al. 2013a). Since then, about 10 clinical trials using stem cells or mononuclear cells to treat autism have been registered in clinicaltrials.gov (Table 7.1). The kinds of cells or/and stem cells used to date include: mononuclear cells from bone marrow (Sharma et al. 2013a, b, 2015, 2016), umbilical cord blood (Lv et al. 2013), fetal stem cells (Bradstreet et al. 2014), and mesenchymal stem cells from umbilical cord (Lv et al. 2013). Transplantation of these cells significantly improved some scores in autistic patients (Bradstreet et al. 2014; Lv et al. 2013; Sharma et al.

Table 7.1 Clinical trials using stem cells for autism treatment

No	Names of study	Phase	Auto/Allo	Status	Country
1	A Clinical Trial to Study the Safety and Efficacy of Bone Marrow Derived Autologous Cells for the Treatment of Autism NCT01836562	I/II	Intrathecal injection of 100 million autologous mononuclear cells (MNCs) from bone marrow in 3 doses at 10 days interval.	Recruiting	India: Institutional Review Board
2	Autologous Bone Marrow Stem Cells for Children With Autism Spectrum Disorders NCT01740869	I/II	Intrathecal injection of autologous $CD34^+$ stem cells from bone marrow	Recruiting	Mexico: Ethics Committee
3	Allogeneic Umbilical Cord Mesenchymal Stem Cell Therapy for Autism NCT02192749	I/II	Allogeneic human umbilical cord tissue-derived mesenchymal stem cells administered intravenously (IV) in a series of 4 infusions every 3 months over the course of 1 year	Active, not recruiting	Panama: Ministry of Health
4	Safety and Efficacy of Stem Cell Therapy in Patients With Autism NCT01343511	I/II	Intrathecal injection of allogenic human cord blood mononuclear cells and human umbilical cord MSCs	Completed	China: Ministry of Health
5	Autologous Bone Marrow Stem Cell Therapy for Autism NCT02627131	II	Transplantation of Autologous Bone Marrow MNCs	Completed	Vietnam: Hanoi Department of Health
6	Autologous Cord Blood Stem Cells for Autism NCT01638819	II	Infusion of autologous umbilical cord blood (AUCB) containing a minimum of 10 million total nucleated cells per kilogram (TNC/kg)	Active, not recruiting	United States: Food and Drug Administration

(continued)

Table 7.1 (continued)

No	Names of study	Phase	Auto/Allo	Status	Country
7	Adipose Derived Stem Cell Therapy for Autism NCT01502488	I/II	Autologous adipose-derived stromal cells delivered intravenously	Not yet recruiting	Mexico: Cofepris
8	Stem Cell Therapy in Autism Spectrum Disorders NCT01974973	I	Autologous bone marrow MNC transplantation	Recruiting	India: Indian Council of Medical Research

2013a; Sharma et al. 2013b). Adipose derived stem cells and bone marrow derived hematopoietic stem cells (HSCs) have also been used in the clinic (Table 7.1). However, their treatment efficacies have not yet been reported. In almost all of the studies, stem cells were delivered to patients by intrathecal injection; a few studies used intravenous infusion.

7.3.2.1 Bone Marrow Stem Cells

The first application of stem cells in ASD was reported in 2013. The case involved a 14-years old boy with severe autism who was treated with autologous bone marrow-derived mononuclear cells (BMMNCs) intrathecally (Sharma et al. 2013a). In this study by Sharma et al. (Sharma et al. 2013a), the authors collected the autologous bone marrow then isolated the mononuclear cells and intrathecally injected them into the patient. After 6 months, the results showed a symptomatic improvement with a shift on the Childhood Autism Rating Scale (CARS), from 42.5 (Severely Autistic) to 23.5 (Non Autistic). As well, an enhanced PET scan brain function was noted (Sharma et al. 2013a).

In the same year, Sharma et al. (Sharma et al. 2013a, b) published their findings from an open clinical trial designed to treat autism through the use of autologous BMMNCs (Sharma et al. 2013b). This study followed the same approach to treat autism as their previous study. All transplantations used autologous cells and were infused intrathecally. Besides transplantation with BMMNCs, all patients were also monitored by multidisciplinary approaches. There were 32 patients in this study who were followed for 26 months after treatment. The study showed that 91 % (29/32 patients) significantly improved on total ISAA scores, 62 % (20/32 patients) significantly decreased severity on CGI-I ($P < 0.001$), and 97 % (31/32 patients) globally improved on CGI-II (Sharma et al. 2013b). Using the same strategy, Sharmar et al. confirmed efficacy of this type of approach in other patients, in 2015 (Sharma et al. 2015) and 2016 (Sharma et al. 2016).

7.3.2.2 Umbilical Cord and Umbilical Cord Blood Stem Cells

The combination of human cord blood mononuclear cells (CBMNCs) and umbilical cord-derived mesenchymal stem cells (UCMSCs) have also been used clinically to treat autism. In a phase I/II clinical study by Lv et al. (2013), 37 patients were divided into 3 groups: 14 patients received CBMNC transplantation and rehabilitation therapy, 9 patients received CBMNC + UCMSC transplantation and rehabilitation therapy, and 14 subjects received only rehabilitation therapy (control group). The study demonstrated that transplantation of the cells was safe, with no adverse effects. The groups treated with cells significantly improved their CARS and CGI evaluation at 24 weeks post-treatment ($p < 0.05$). In addition, they improved their Aberrant Behavior Checklist (ABC) score- for domains of speech, sociability, sensory, and overall health. The study also showed that the combination of CBMNCs and UCMSCs provided a greater therapeutic efficacy than CBMNC transplantation alone (Lv et al. 2013).

7.3.2.3 Fetal Stem Cells

In a recent clinical trial with an open-labeled pilot study, Bradstreet et al. (2014) used fetal stem cells (FSCs), which possess high immunoregulatory functions, as MSCs to treat ASD, which were regarded as autoimmune diseases (Bradstreet et al. 2014). The study involved transplantation of 2 injections of FSCs (one given intravenously and the other given subcutaneously) in children diagnosed with ASDs. The patients were monitored pre-treatment, and at 6 and 12 months post transplantation. The results showed that FSC transplantation had no adverse events in the ASD patients; no transmitted infections or immunological complications were observed. These treated patients showed significant improvement on the Autism Treatment Evaluation Checklist (ATEC) test. As well, they showed improvement on the ABC score and reduction in total scores when compared to pre-treatment values (Bradstreet et al. 2014).

7.4 The Future

With increasing studies, the pathophysiology and disease mechanism of autism is becoming clearer. More and more evidence show that autism is related to malfunction of the immune system; i.e. autism is an autoimmune disease. Thus, stem cells are attractive candidates for treatment of autism and ASDs. Since ASDs can be regarded as autoimmune diseases, there are 3 popular ways to potentially treat ASDs: immune correction, immune modulation, and immune replacement (gene correction) (Fig. 7.1).

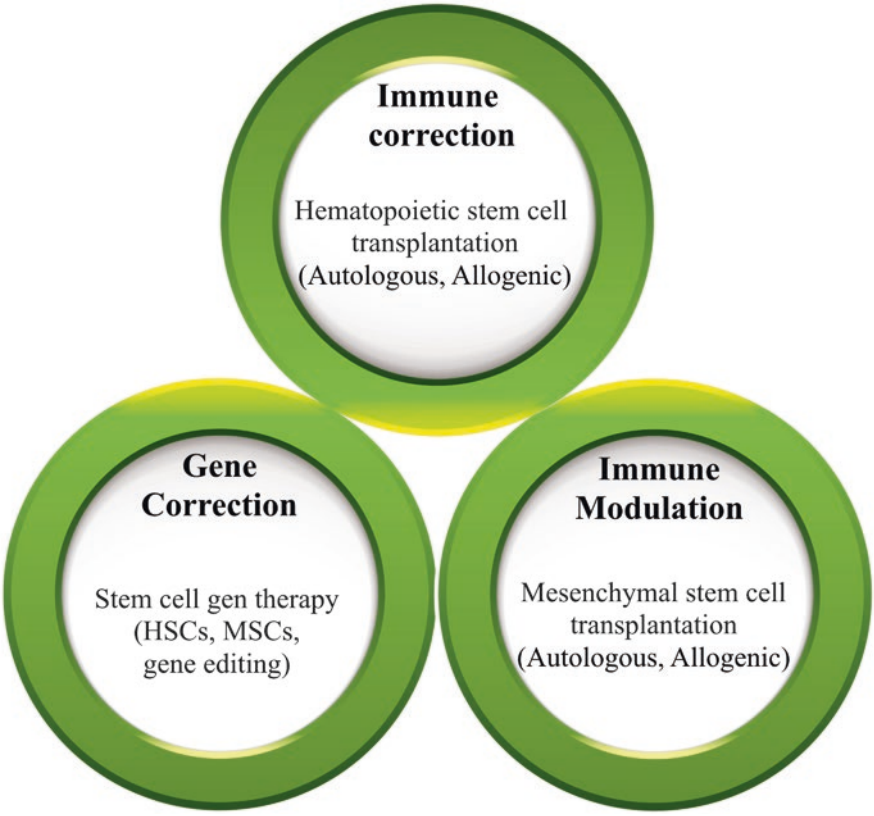

Fig. 7.1 Three approaches based on stem cells for autoimmune diseases. Autoimmune diseases can be treated by three approaches: immune correction, immune modulation, and gene correction. Immune system can be corrected by HSC transplantation, while immune modulation usually can be facilitated by MSCs. The third approach related to gene correction has also been studied, and can involve both HSCs and MSCs

7.4.1 Immune Modulation (Immunomodulation)

Immune modulation (also called immunomodulation) is related to the capacity of MSCs to modulate the activity and function of the immune system. In some cases, immunomodulation is similar to immune suppression. Unlike immune suppression drugs, MSCs can modulate the immune system by suppressing some immune cells, while stimulating other immune cells. In contrast to other stem cells, MSCs have a remarkable capacity to regulate immune responses. Many studies have shown that MSCs can regulate immune responses both *in vitro* and *in vivo*. MSCs have been demonstrated to mainly affect 4 kinds of immune cells: T lymphocytes (Aggarwal

and Pittenger 2005; Di Nicola et al. 2002; English et al. 2009), B lymphocytes (Asari et al. 2009; Augello et al. 2005; Corcione et al. 2006), NK cells (Sotiropoulou et al. 2006; Spaggiari et al. 2006), and dendritic cells (DCs) (Chen et al. 2007; Zhang et al. 2004). With regard to T lymphocytes, MSCs can suppress T-cell proliferation induced by cellular or nonspecific mitogenic stimuli (Di Nicola et al. 2002), alter the cytokine secretion profile of naïve and effector T cells (Aggarwal and Pittenger 2005), and promote the expansion and function of regulatory (Treg) cells (English et al. 2009). Regarding B lymphocytes, MSCs can also inhibit their proliferation (Augello et al. 2005), affect their chemotactic properties (Corcione et al. 2006), and suppress their terminal differentiation (Asari et al. 2009). For NK cells, MSCs can alter their phenotype, and suppress their proliferation, cytokine secretion, and cytotoxicity against targets expressing HLA class I (Sotiropoulou et al. 2006; Spaggiari et al. 2006). Finally, for DC cells, MSCs can influence the differentiation, maturation, and function of monocyte-derived DCs (Zhang et al. 2004), suppress DC migration, maturation and antigen presentation (Chen et al. 2007), and induce a novel Jagged-2-dependent regulatory DC population (Zhang et al. 2009) (Fig. 7.2).

With these properties, MSCs have been increasingly studied for autoimmune disease treatment. The autoimmune diseases have included preclinical and clinical trials of systemic lupus erythematosus (SLE) (Gu et al. 2014; Wang et al. 2014; Yan et al. 2013), Crohn's disease (Ciccocioppo et al. 2015; Liew et al. 2014), multiple system atrophy (MSA) (Lee et al. 2012; Sunwoo et al. 2014), multiple sclerosis (MS) (Dulamea 2015; Gharibi et al. 2015), and amyotrophic lateral sclerosis (ALS) (Hajivalili et al. 2015; Lewis and Suzuki 2014; Rushkevich et al. 2015).

Moreover, MSCs are adult stem cells which can be isolated from various sources, including adipose tissue (Zuk et al. 2001, 2002), peripheral blood (Fernandez et al. 1997; Huss et al. 2000; Purton et al. 1998), umbilical cord blood (Erices et al. 2000; Lee et al. 2004; Mareschi et al. 2001), banked umbilical cord blood (Phuc et al. 2012; Phuc et al. 2011), umbilical cord (Kestendjieva et al. 2008; Romanov et al. 2003), umbilical cord membrane (Kita et al. 2010), Wharton's jelly of the umbilical cord (Hou et al. 2009), placenta (Rylova et al. 2015), and dental pulp (Jo et al. 2007; Pierdomenico et al. 2005). The availability of MSCs from different sources have made MSCs a valuable cell platform for the treatment of autism. Various preliminary studies have indicated that MSCs can potentially improve autism (Lv et al. 2013).

7.4.2 Immune Correction

Another approach used to treat autoimmune disease is immune correction. Immune correction is mediate by HSC transplantation, which helps to produce new immune cells from autologous HSCs. The exact mechanism for this approach remains unclear. However, suitable explanations have been suggested. In the autoimmune disease patients, autoreactive effector and inflammatory cells produced by the

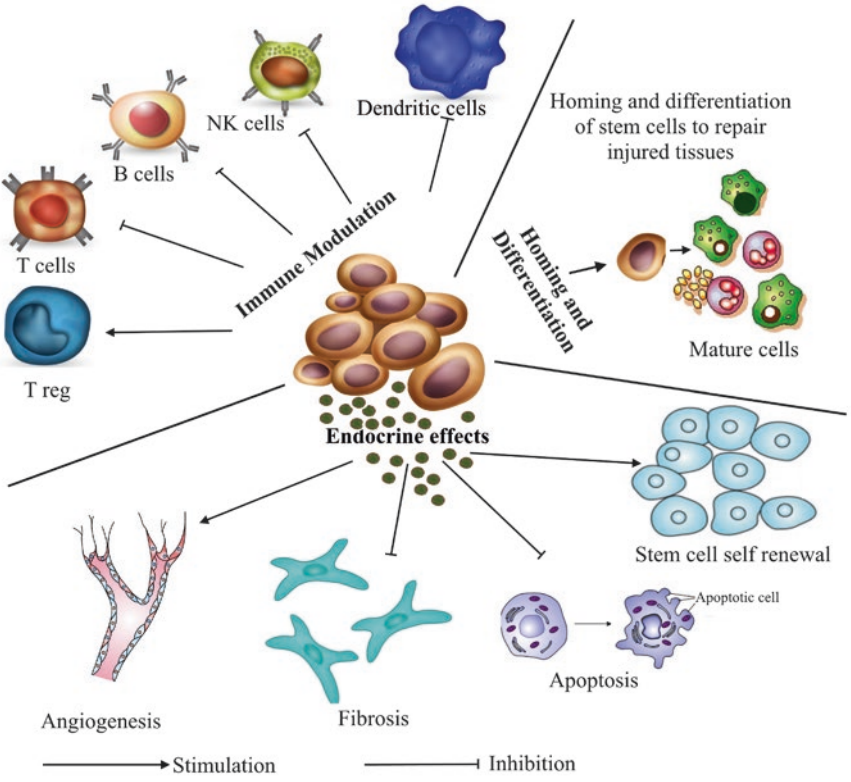

Fig. 7.2 Some mechanisms of MSCs for treating autoimmune diseases. To date, there are at least three ways MSCs can act on autoimmune diseases. (1) MSCs can modulate the host's immune system; (2) MSCs can home to injured tissue and differentiate into specific cells that replace the injured cells at those tissues; (3) MSCs release a variety of cytokines and growth factors that can inhibit fibrosis and apoptosis at injured tissues, trigger the self-renewal process of stem cells, and stimulate angiogenesis

immune system attack auto-tissues/cells and cause the autoimmune reaction. Via HSC transplantation, these autoreactive or inflammatory cells are removed and freshly transplanted HSCs can reset the immune system of the patient. Presumably, after HSC transplantation, the de novo immune cells in the new immune system will not produce autoreactive effector and inflammatory cells like before.

The outcome of autologous HSC transplantation are: (i) an increased number of regulatory, FoxP3-positive T cells (Roord et al. 2008), (ii) the reactivation of thymic function which leads to a tolerant, "juvenile" immune system (Alexander et al. 2009; Muraro et al. 2005), and (iii) antithymocyte globulin directly targets long-living, autoantibody-producing plasma cells by complement-mediated lysis and apoptosis (Zand et al. 2006).

Indeed, HSC transplantation was used in 1997 to treat the first autoimmune diseases, including severe and therapy-refractory autoimmunity (Tyndall et al. 1997).

The European Bone Marrow Transplantation (EBMT) database, PROMISE, is the largest database of transplanted patients with autoimmune diseases and currently includes data on more than 1,000 patients from 172 institutions in 27 countries. Pilot studies were conducted for systemic sclerosis (Martini et al. 1999; Tyndall et al. 1997), rheumatoid arthritis (Durez et al. 1998), SLE (Burt et al. 1998a, b; Marmont et al. 1997), multiple sclerosis (Burt et al. 1998a), and hematological autoimmune diseases (e.g. idiopathic thrombocytopenia (Lim et al. 1997), autoimmune hemolytic anemia, and Evans syndrome). The studies showed that HSC transplantation achieved improvement in patients with all the above conditions.

7.4.3 Immune Replacement/Gene Correction

Similar to other autoimmune diseases, there have been gene mutations detected and linked to autism (Durand et al. 2007; Jamain et al. 2003; Laumonnier et al. 2004; Neale et al. 2012; Sebat et al. 2007). Immune replacement or gene correction relates to replacing the mutated immune system with a healthy immune system. Theoretically, there are two ways to correct the immune system: allogeneic HSC transplantation that uses healthy HSC donor with HLA matching or correction of gene mutations in the patient's HSCs with gene engineering. Both strategies are, however, limited and difficult to perform. The development of induced pluripotent stem cells, and especially the technology of differentiation of induced pluripotent stem cells (iPSCs) into HSCs, has opened a new direction in immune correction and proven potentially promising (Lachmann et al. 2015; Lim et al. 2013).

Autoimmune diseases where iPSCs from patients have been successfully produced include systemic lupus erythematosus (Chen et al. 2013), multiple sclerosis (Fossati and Douvaras 2014), and type 1 diabetes (Liu et al. 2014). In a recent study, Son et al. (2016) were able to produce iPSCs from patient-derived cells. Using the nonintegrating oriP/EBNA-1-based episomal vectors, iPSCs from ankylosing spondylitis (AS), Sjögren's syndrome (SS) and systemic lupus erythematosus (SLE) patients have been successfully generated. Interestingly, these iPSCs could differentiate into cells of hematopoietic and mesenchymal lineages *in vitro* (Son et al. 2016). These results provide a renewed hope in the near future for treating autoimmune diseases, generally, and autism, specifically, via immune replacement (Fig. 7.3).

7.5 Conclusion

Autism is a complex disease and is, therefore, referred to as ASDs. Some recent discoveries of ASD pathophysiology and mechanisms have revealed that autism is an immune disease with some characteristics of autoimmune diseases. ASDs have been recently treated with stem cells; the results show that stem cell therapy is

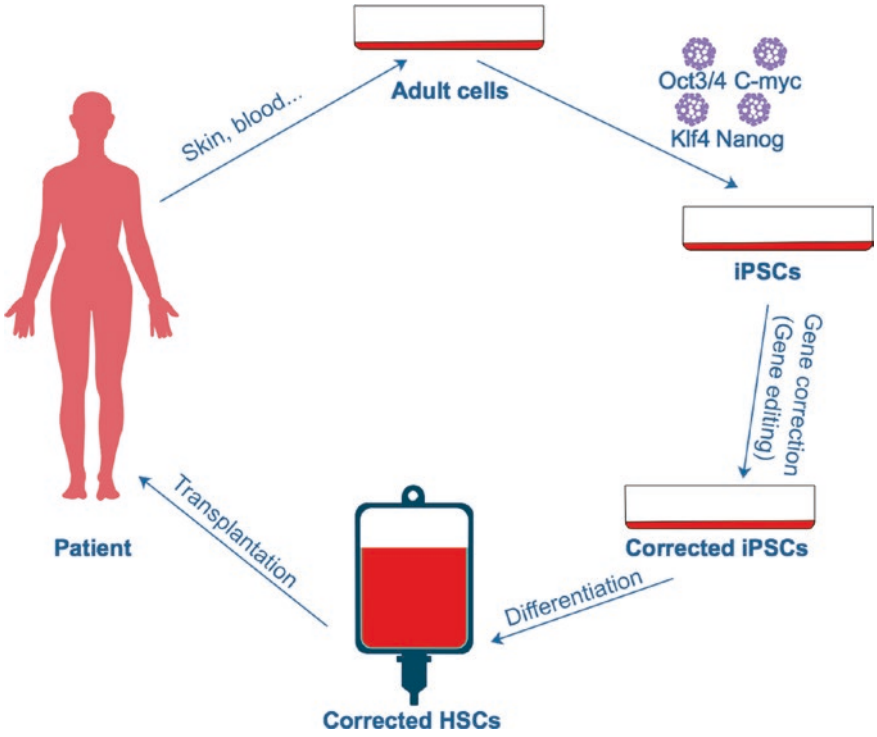

Fig. 7.3 Immune correction strategy by reprogramming in combination with gene editing. Adult cells such as skin cells, blood cells… were collected from the patients. Then they were reprogrammed into induced pluripotent stem cells (iPSCs) by some vectors such Oct-3/4, Nanog, c-Myc, Klf4. These iPSCs were corrected mutated genes by gene editing. Finally, these corrected iPSCs were differentiated into hematopoietic stem cells for treatment

beneficial for autism patients. Although to date, few clinical studies utilized stem cells, a small patient population have been treated with stem cells. The promising results lend rationale for the use of stem cell therapy for autism in the near future.

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Chapter 8
Stem Cell Therapy for Spinal Cord Injury

Sicong Tu and Jian Tu

8.1 Introduction

Spinal cord injury remains a leading cause of long-term disability worldwide, resulting in enormous losses to individuals, families, and communities (WHO 2013a). World Health Organization has estimated that 500,000 people suffer a spinal cord injury each year. People with spinal cord injuries are two to five times more likely to die prematurely than people without a spinal cord injury (WHO 2013a). Up to 90 % of spinal cord injury cases are due to traumatic causes such as road traffic crashes, falls and violence (WHO 2013a). Symptoms of spinal cord injury may include partial or complete loss of sensory function or motor control of arms, legs, and/or body. The most severe spinal cord injury affects the systems that regulate bowel or bladder control, breathing, heart rate and blood pressure (WHO 2013b). Most people with spinal cord injury experience chronic pain, and an estimated 20–30 % show clinically significant signs of depression. People with spinal cord injury also risk developing secondary conditions that can be debilitating and even life-threatening, such as deep vein thrombosis, urinary tract infections, pressure ulcers and respiratory complications (WHO 2013a).

However, no effective therapy is available for treatment of individuals with spinal cord injury; nonetheless, researchers had tried some therapeutic agents like levodopa (Maric et al. 2008) and some neurotrophic factors in spinal cord injury (Cao and Dong 2013; Blesch et al. 2012; Boyce and Mendell 2014). This needs experimentation to confirm if these dopamine precursors and neurotrophic factors have any role in the treatment of spinal cord injury. Several other therapeutic agents like erythropoietin (Baptiste and Fehlings 2006), cannabinoid dexanabinol (McConeghy et al. 2012), and gamma-glutamylcysteine ethyl ester (Boyd-Kimball

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et al. 2005) have all shown to have neuroprotective effect in human at experimental stage with remarkable improvement in post-traumatic spinal cord injury outcome.

Recent advancement in knowledge about stem cells promotes stem cells therapy in spinal cord injury. The stem cells may play an important role in the treatment of spinal cord injury by replacing damaged cells, and helping long-term functional recovery (Donnelly et al. 2012; Zhao et al. 2013; Davies et al. 2011). The search for stem cell therapy for human spinal cord injury is promising and progressing (Perrin et al. 2010). One obstacle in the search for an effective stem cell therapy is that the pathophysiology of spinal cord injury is largely unknown. This is because multiple cell types like neuronal cells, glial, and endothelial cells are usually involved in spinal cord injury. Furthermore, the vasculature of the spinal cord, especially the blood spinal cord barrier may be affected in spinal cord injury; this injury may be focal or diffuse axonal injury. This often results in neuronal mitochondrial dysfunction as we recently reported (Hu 2015). Taming these burgeoning effects of spinal cord injury requires neural stem cells that can differentiate into functional neurons and glial cells. We have reported that progenitor cells differentiated into neurons and glial in adult spinal cord, and an increase in astrocytic progeny forming reactive astrocytes to primarily limit cyst enlargement in posttraumatic syringomyelia (Tu et al. 2011, 2010).

This chapter is an optional extra to confirm whether we can achieve the translation of basic knowledge of neural stem cells into therapeutic options in persons with spinal cord injury by enhancing and integrating these neural precursor cells unto neurogenesis and directing these cells to the specified targets or through multipotency where the transplanted stem cells can differentiate into glial cells, neurons, and endothelial cells. As spinal cord injuries are not always focal but diffuse we need to induce these transplanted stem cells differentiating into appropriate phenotype for long-term structural and functional recovery. This chapter critically reviews current literature of others and our previous reports on neural stem cell research and proposing an approach for the quality clinical translation of stem cell research to therapy in spinal cord injury. The author explains the pathophysiology of spinal cord injury and proposes the “six-point schematic approach” to achieving quality bench to bedside translation of neural stem cells to therapy for spinal cord injury. The author also highlights the need for suitable clinical translation, coordination, and administration of research in the field of stem cell therapy for spinal cord injury.

8.2 Neuropathology of Spinal Cord Injury

The term ‘spinal cord injury’ refers to damage to the spinal cord resulting from trauma (e.g. a car crash) or from disease or degeneration (e.g. cancer) (WHO 2013a). Up to 90 % of these cases are due to traumatic causes (WHO 2013a). Therefore, neuropathology of traumatic spinal cord injury is focused below. Pathophysiology of traumatic spinal cord injury involves two main phases (Nakamura et al. 2003). These are the primary spinal cord injury following the trauma, and the secondary

injury that are mediated by the inflammatory response to the primary spinal cord injury.

8.2.1 Primary Injury After Traumatic Spinal Cord Injury

Neuropathology of the initial spinal cord injury can be categorized as acute impact or compression (Kraus 1996). Acute impact injury is a concussion of the spinal cord. These inertial forces disrupt the blood-spinal cord barrier (BSCB). This type of injury initiates a cascade of events focused on the gray matter and results in haemorrhagic necrosis. The initiating event is a hypoperfusion of the gray matter. The primary events also involve massive ionic influx referred to as traumatic depolarization. Increases in intracellular calcium and reperfusion injury play key roles in cellular injury and occur early after injury. The extent of necrosis is contingent on the amount of initial force of trauma but also involves concomitant compression, perfusion pressures, and blood flow. The major inflammatory neurotransmitters released from the damaged tissue are excitatory amino acids, which may explain the neuropathology of diffuse axonal injury in traumatic spinal cord injury. Spinal cord compression occurs when a mass impinges on the spinal cord causing increased parenchymal pressure. This occurs in the white matter, whereas gray matter structures are preserved. Rapid or a critical degree of compression will result in the collapse of the venous side of the microvasculature, resulting in vasogenic edema. Vasogenic edema exacerbates parenchymal pressure, and may lead to rapid progression of spinal cord dysfunction (Kraus 1996). The expression of high levels of glucose transporter 1 was observed in capillaries from acutely injured the spinal cord, which occurs in association with compromised blood-spinal cord barrier function. Vascular endothelial growth factor also plays a role in neuronal tissue disruption and increases the permeability of the blood-spinal cord barrier via the synthesis and release of nitric oxide. Figure 8.1 depicts the neuropathology of the primary injury after traumatic spinal cord injury.

8.2.2 Secondary Injury After Traumatic Spinal Cord Injury

The secondary events are a complex association of the inflammatory response initiated by the trauma leading to diffuse neuronal degeneration of neurons, glial, axonal tearing, and genetic predisposition (Fig. 8.2). There are significant increases in cytokine (IL-1alpha and IL-1beta) and chemokine (MCP-1, GRO/KC, and MIP-1alpha) production (Bastien and Lacroix 2014; de Rivero Vaccari et al. 2014), MPO activity, blood-spinal cord barrier (BSCB) permeability, and MMP-9 activity in the damaged spinal cord (Austin et al. 2012; Anthony and Couch 2014). Furthermore, excitatory amino acids release, oxygen radical reactions, and nitric oxide production lead to the activation of N-methyl-D-aspartate,

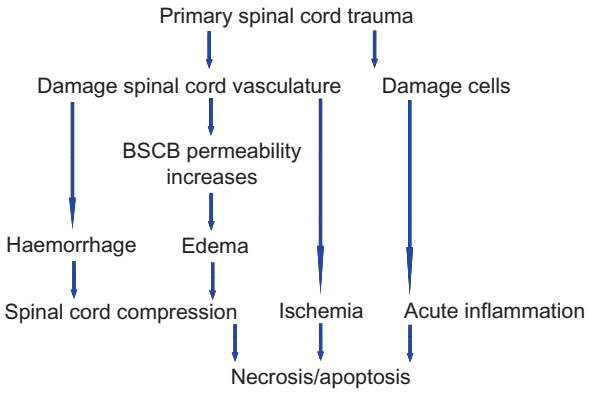

Fig. 8.1 Sequential events of primary spinal cord injury. Initial impact is usually by direct trauma to the spinal cord. This trauma will cause mechanical damage to neurons, axons, glia and blood vessels by shearing, tearing or stretching. Blood vessel ruptures cause haemorrhage. Even in unruptured blood vessels, the blood-spinal cord barrier permeability increases resulting in edema. Haemorrhage and edema often lead to spinal cord compression. Following haemorrhage, ischemia could occur in the spinal cord tissue. Traumatic spinal cord damages caused cell damage induces macrophage and lymphocytes migrant to the injury site releasing inflammatory mediators that triggers a cascade of events towards necrosis and/or apoptosis. Necrosis and/or apoptosis also can be a consequence of haemorrhage and ischemia

2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)propanoic acid, alpha-7 nicotinic receptor (α7), and nicotinic acetylcholine receptor and subsequent calcium influx. All these cascades of events cause mitochondrial disruption (Hu 2015) and free radical release with eventual tissue peroxidation. One theory is that excitatory amino acid release leads to calcium influx into neurons and other cells which promote oxygen free radical reactions. High calcium and the presence of free-radical molecules create an unstable environment in the neurons that lead to increased production and release of nitric oxide and excitatory amino acids, such as glutamate. Nitric oxide participates in oxygen radical reactions and lipid peroxidation in neighboring cells. A summary of the secondary injury after traumatic spinal cord injury is shown in Fig. 8.2. The secondary injury plays a major role in the outcome of traumatic spinal cord injury. Demyelinated axons are vulnerable to degeneration; without rapid remyelination, the neurons may die, resulting in worsened damage and functional impairment. Therapeutic interventions should target this phase as it is the major determinant of morbidity and mortality in traumatic spinal cord injury. Clinically, the application of stem cell therapy early to patients with traumatic spinal cord injury is ethically challenging because of the difficulty in obtaining informed consent immediately following the spinal cord injury. Genes implicated to influence the outcome of traumatic spinal cord injury (Nishimura et al. 2014) include *Sox11* (Wang et al. 2015), *apoe* (Wang et al. 2014), *ace*, and *cacna1a. Sox11* gene encodes a member of the SOX (SRY-related HMG-box) family of transcription factors whose expression is common to a many types of regenerating neurons. Sox11 reduces axonal dieback of dorsal root

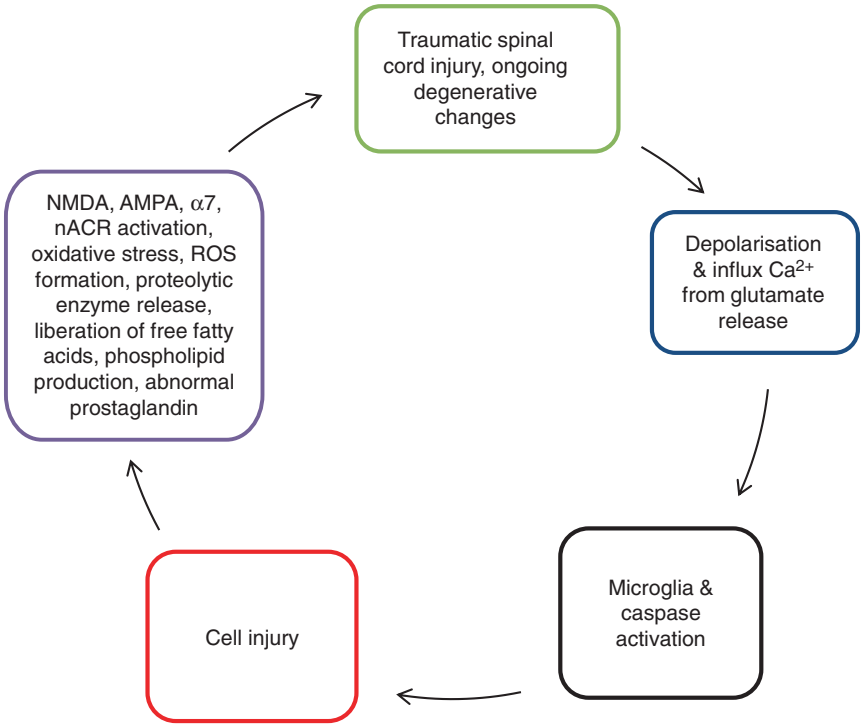

Fig. 8.2 Sequential events of secondary damages in traumatic spinal cord injury. This includes a variety of processes including depolarization, disruption of ionic homeostasis and release of neurotransmitters, lipid degradation, and oxidative stress. These events are a result of interaction between the excitatory amino acids released with an influx of oxygen free radicals that ultimately set up NMDA, AMPA, α7 and nACR to sustain the unstable environment for cell injury and degenerative changes

ganglion axons and promotes corticospinal tract sprouting and regenerative axon growth in both acute and chronic injury paradigms (Wang et al. 2015). *Apoe* multifactorially affects the clinicopathological consequences of traumatic spinal cord injury (Resnick et al. 2004; Wang et al. 2014). *Apoe* gene encodes apolipoprotein E and is associated with increased amyloid deposition, amyloid angiopathy, larger intracranial haematomas, and more severe contusional injury. The *ace* gene encodes angiotensin-converting enzyme (EC 3.4.15.1) (Niu et al. 2002) and affects traumatic spinal cord injury outcome *via* alteration of spinal blood flow and/or autoregulation. The *cacna1a* gene encodes the alpha subunit of neuronal Ca2.1 Ca^{2+} channels (van den Maagdenberg et al. 2004) and exerts an influence *via* the calcium channel pathways and its effect on delayed vasogenic edema. Increased signal transducers and activator of transcription 3 signaling have been reported in a rodent model of traumatic spinal cord injury. Although several potential genes that may influence the outcomes following traumatic spinal cord injury have been identified, future investigations are needed to validate these

genetic studies (Resnick et al. 2004), and identify new genes that might contribute to the patient outcomes after traumatic spinal cord injury.

8.3 Current Pharmacotherapy for Spinal Cord Injury

Pharmacotherapies aim at promoting neurorepair, neuroregeneration, and neuroprotection following traumatic spinal cord injury. Gold standard therapy for SCI has yet established (Bydon et al. 2014; Cao and Dong 2013), although clinical trials with 17beta-estradiol and progesterone (Elkabes and Nicot 2014), the sodium (Na+) channel blocker Riluzole (Wu et al. 2014; Wilson and Fehlings 2014; Grossman et al. 2014), methylprednisolone (NASCIS II and III), and GM-1 ganglioside (Maryland and Sygen) have demonstrated modest, albeit potentially important therapeutic benefits (Baptiste and Fehlings 2006). In light of the overwhelming impact of SCI on the individual, other therapeutic interventions are urgently needed. A number of promising pharmacological therapies are currently under investigation for neuroprotective capacities in animal models of SCI. These include the tetracycline derivative Minocycline (Chew et al. 2014; Casha et al. 2012; Monaco et al. 2013; Wilson et al. 2013), the fusogen copolymer polyethylene glycol (PEG), the tissue-protective hormone erythropoietin (EPO) (Baptiste and Fehlings 2006), paired immunoglobulin-like receptor B (PirB) (Gou et al. 2014), chondroitin sulphate proteoglycans (Lang et al. 2015), peroxynitrite (Xiong and Hall 2009), and modulation of the adaptive immune response via active and passive vaccination (Jones 2014). Moreover, clinical trials investigating the putative neuroprotective and neuroregenerative properties ascribed to the Rho pathway antagonist, Cethrin (BioAxone Therapeutic, Inc.), and implantation of activated autologous macrophages (ProCord; Proneuron Biotechnologies) in patients with thoracic and cervical SCI are now underway. Clinical trials evaluating these interventions apply standardized clinical outcome measures to demonstrate efficacy. In the past, drug research and development for traumatic spinal cord injury focused on limiting secondary spinal cord injury after the initial traumatic event because of lacking evidence that the central nervous system could be repaired or regenerated. Growing body of evidence indicates that the adult spinal cord can be repaired and regenerated after traumatic spinal cord injury (Stenudd et al. 2015). Potential drug targets for post-traumatic injury spinal cord repair include angiogenesis, axon guidance and remodeling, remyelination, neurogenesis, and synaptogenesis. Pharmacotherapies may also target spinal cord regeneration by enhancing the capacity of pluripotent cells to differentiate into neurons, glia, and vascular endothelium. Spinal cord repair and regeneration processes can be activated or enhanced by pharmacotherapy over a longer therapeutic window than pharmacologic interventions designed to limit injury. Pharmacotherapies are potentially effective in the acute, subacute, post-acute, and chronic phases after traumatic spinal cord injury (Fig. 8.3). Thus, repair and regeneration therapies have the potential advantage of being effective over a prolonged period of time following traumatic spinal cord injury.

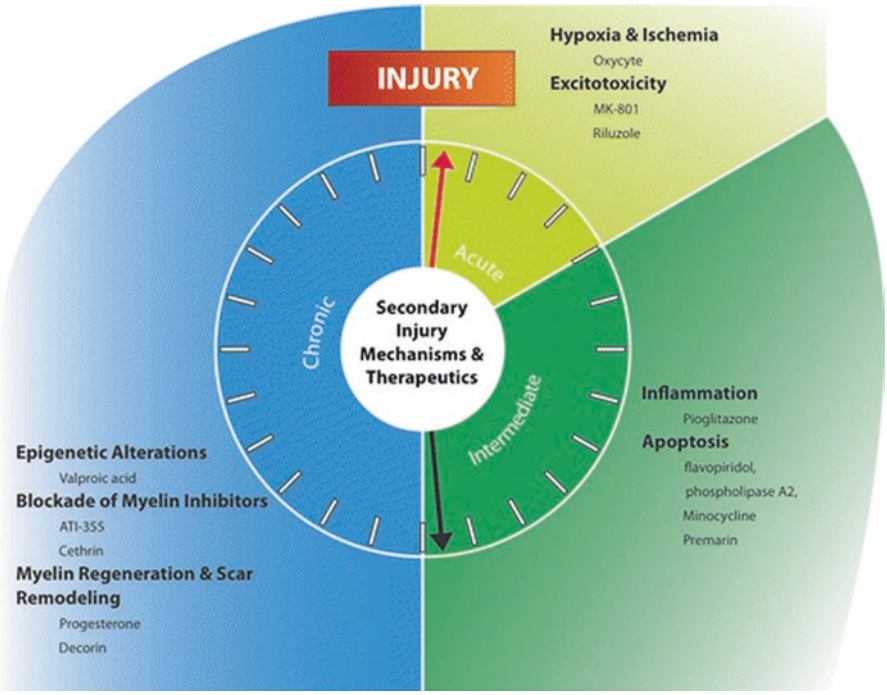

Fig. 8.3 Schematic representing the acute, intermediate, and chronic phases of secondary spinal cord injuries with cognate pathophysiologies and therapeutics. The secondary injury cascade begins within seconds of the primary injury and results in further tissue damage, cell death, inflammation, Wallerian degeneration and glial scarring. Immediately following a traumatic spinal cord injury disruption of blood flow occurs resulting in hypoxia to the injured tissue. Oxycyte, an oxygen carrier can be intravenously injected at the earliest possible time point following an acute injury to increase oxygen availability in damaged tissue and lessen the detrimental cascade triggered by hypoxia. At the acute phase, the released glutamate results in excitotoxicity. MK-801, a glutamate receptor N-Methyl-D-Aspartate (NMDA), and Riluzole, a glutamate receptor modulator can modulate excitotoxicity. Excitotoxicity increases inflammation. Pioglitazone, a synthetic agonist of the ligand-activated transcription factor peroxisome proliferator-activated receptor-gamma (PPARγ) can regulate inflammation. Cellular stressors trigger release of pro-apoptotic signaling molecules. Flavopiridol, a cell cycle inhibitor, and Phospholipase A2 (PLA2), a lipolytic enzyme can reduce both neuronal and oligodendrocyte apoptosis. Minocycline and Premarin (a cocktail of equine conjugated estrogens) can decrease apoptosis. At the chronic phase, epigenetics may limit the central nervous system's ability to regenerate. Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, can reduce gliosis. Myelin inhibitors, such as NoGo, inhibit regeneration of axons. ATI-355, a humanized anti-Nogo antibody, and Cethrin, a recombinant protein RHO GTPase antagonist, can modulate axon regeneration. Progesterone suppresses gliosis at the early stage of spinal cord injury while promotes oligodendrocyte differentiation and remyelination at the later stages (Cox et al. 2015) (Reuse license number 3626490078177 including electronic rights obtained from Springer)

Since 2005, only Cethrin, a recombinant RHO GTPase antagonist, has received approval from the U.S. Food and Drug Administration (FDA) approved as an orphan drug for the treatment of patients with acute cervical and thoracic spinal injuries

(Fehlings et al. 2011; Cox et al. 2015). Table 8.1 lists candidate compounds currently undergoing clinical evaluation for traumatic spinal cord injury treatment. Because traumatic spinal cord injury damages the spinal cord tissue by multiple mechanisms, combination therapy designed to simultaneously target multiple mechanisms of injury is likely required. There is now a growing field of non-pharmacological interventions such as stem cell transplantation, gene therapy, RNAi, and electrical stimulation. Therein a particular emphasis is placed on stem cell therapy that offers an alternative option for traumatic spinal cord injury treatment.

8.4 Stem Cell Therapy in Spinal Cord Injury

There are at least two strategies involving stem cell therapy to repair injured spinal cord tissue. They are transplantation of exogenous stem cells to replace damaged cells and stimulation of endogenous stem cells to proliferate to the number of cells needed and differentiate them to the phenotype of cells required for normalization of spinal cord function.

8.4.1 Transplantation of Exogenous Stem Cells for the Treatment of Spinal Cord Injury

There is considerable number of attempts to transplant various types of cells, such as embryonic postmitotic motoneurons, neural precursor cell, embryonic stem cells, neural stem cells, astrocytes, oligodendroglia cells, umbilical cord blood stem cells, adipose-derived stem cells, dental mesenchymal stem cells, and exfoliated deciduous teeth-derived stem cells to repair damaged spinal cord tissue (Aftab et al. 2013; Das et al. 2011; Davies et al. 2011; Donnelly et al. 2012; Hewson et al. 2013; Kabatas and Teng 2010; Kolar et al. 2014; Ning et al. 2013; Nogradi et al. 2011; Perrin et al. 2010; Taghipour et al. 2012; Volarevic et al. 2013; Xiao and Tsutsui 2013; Yamada et al. 2014). The main objectives of transplantation experiments are (1) growth facilitation: the transplant fills the lesion site and serves as a cellular bridge; (2) new neurons: the transplant can provide new neurons, which in turn provide new targets and sources of innervations and thus repair the damaged neural circuits; (3) factor secretion: the transplant can produce a variety of substances, such as neurotrophic factors, that promote the spinal cord tissue repair process (Barami and Diaz 2000). Several characteristics of neural stem cells make them potentially suitable to repair damaged spinal cord tissue after traumatic spinal cord injury. Firstly, they can serve as a renewable supply of transplantable cells by clonal expansion in cell culture. Secondly, they are of central nervous system origin, and the stem cells generated from the grafts have neural characteristics. Thirdly, neural stem cells can be manipulated by genetic engineering methods to produce specific proteins, such as neurotrophins, neurotransmitters and enzymes (Taha 2010).

Table 8.1 Pharmacotherapies undergoing clinical evaluation for spinal cord injury

Compound	Neuroprotective mechanism	Preclinical evidence	Clinical evidence	References
Cethrin	Modulates axon regeneration in SCI, Enhances motor function recovery	Recombinant RHO GTPase antagonist, Blockade of myelin inhibitors	FDA approved orphan drug to treat acute cervical & thoracic spinal injuries; Undergoing efficacy trial	Fehlings et al. (2011)
ATI-355	ibid	Humanized anti-Nogo antibody, Blockade of myelin inhibitors	Results from the trials have not been released	Cox et al. (2015)
Riluzole	Modulates excitotoxicity	A sodium channel blocker/glutamate receptor modulator	Enhanced improvement in motor score in phase I trial; Undergoing efficacy trial	Grossman et al. (2014)
Growth hormone	Has neuroprotective & neuroregenerative effects	Improves motor function, Corrects impairments of endothelial progenitor cells, Anti-apoptosis	FDA-approved for adult patients with acquired growth hormone deficiency	Behrman et al. (1995)
Estrogen	Improved locomotor function recovery, Neuroprotective effects	Anti-inflammatory, Antioxidant, Promotes angiogenesis, Preserves oligodendrocyte, Modulates excitotoxicity	Undergoing safety trial of 5 patients with ASIA A or B grade injuries	Cox et al. (2015), Samantaray et al. (2011)
Minocycline	Improved motor function	Anti-apoptosis	FDA approved fast-tracking drug; Improved motor function in phase II trial; Undergoing a phase III multi-center efficacy trial	Casha et al. (2012), Teng et al. (2004), Wells et al. (2003)

The efficacy of transplantation largely depends on a grafting method that optimizes the survival of the transplanted stem cells and minimizes the graft-induced lesion. Most transplantation studies involved intraparenchymal injection into the central nervous system, in which cells were grafted directly into or adjacent to the lesion (Chow et al. 2000; Cao et al. 2001; Jendelova et al. 2004). The optimal time for transplantation may not be immediately after injury. The levels of various inflammatory cytokines (tumor necrosis factor-alpha, interleukin-1α, interleukin-1β and interleukin-6) in the injured brain peak 6–12 h after injury and remain elevated until the 4th day. Although these inflammatory cytokines are known to have both neurotoxic and neurotrophic effects, they are believed to be neurotoxic within a week after injury, which causes the microenvironment to be unsuitable for survival of the grafted stem cells (Zhu et al. 2006). However, if too much time passes after the injury, a glial scar forms a barrier surrounding the lesion site and inhibits revascularization of the graft preventing local blood circulation that is needed for graft survival. Thus, it is considered those 7–14 days after traumatic spinal cord injury is the optimal time for stem cell transplantation (Ogawa et al. 2002; Okano et al. 2003). Keirstead et al. compared the transplantation of human embryonic stem cell (hESC)-derived oligodendrocyte progenitor cells (OPCs) into adult rat spinal cord injuries 7 days or 10 months after injury (Keirstead et al. 2005). In both cases, transplanted cells survived, redistributed over short distances and differentiated into oligodendrocytes. Animals that received OPCs 7 days after injury exhibited enhanced remyelination and substantially improved locomotor ability. In contrast, when OPCs were transplanted 10 months after injury, there was no enhanced remyelination or locomotor recovery (Keirstead et al. 2005).

The Food and Drug Administration (FDA) approved the first clinical trial using human embryonic stem cells to treat spinal cord injury in the United States on January 23, 2009 (Alper 2009). Geron Corporation (Menlo Park, California, U.S.A.) developed a product, GRNOPC1 derived from human embryonic stem cells, stimulating nerve growth in patients with debilitating damage to the spinal cord (Alper 2009), according to the test results of human embryonic stem cell (hESC)-derived oligodendrocyte progenitor cells (OPCs) in animals (Keirstead et al. 2005). Geron enrolled four patients suffering from spinal cord injuries to participate in the trial. The first patient, Timothy J. Atchison was treated at the Shepherd Center (Atlanta, GA, U.S.A.) just 2 weeks after he sustained a spinal cord injury in a car accident. Following one injection of GRNOPC1 containing approximately two million cells, Atchison "has begun to get some very slight sensation: He can feel relief when he lifts a bowling ball off his lap and discern discomfort when he pulls on hairs on some parts of his legs. He has also strengthened his abdomen" (Stein 2011). The preliminary results from the clinical trial suggested that the participants experienced no serious adverse events. In addition, no changes in the spinal cord or neurological condition were found. The trial discontinued in November 2011 because of business reasons (Stein 2011). After Geron's stem cell assets had been acquired by BioTime in 2013, BioTime indicated that it plans to re-start the embryonic stem cell-based clinical trial for spinal cord injury (Brown 2013).

8.4.2 *Stimulation of Endogenous Neural Precursor Cells in Spinal Cord Injury*

Endogenous neurogenesis has been identified in adult spinal cord (Tu et al. 2010, 2011). The discovery of endogenous neural stem cells in the adult spinal cord has raised the hope for future noninvasive therapy for spinal cord injury. Neural stem cells have unique self-renewal and multipotent differentiation capacities. Endogenous cell proliferation and gliogenesis have been extensively documented in spinal cord injury, particularly in terms of proliferating oligodendrocyte progenitor cells (McTigue and Sahinkaya 2011; Stenudd et al. 2015). Ependymal cells are ciliated cells lining the central canal of the spinal cord. They are responsible for the propulsion of cerebrospinal fluid and function as a barrier to the spinal cord parenchyma. Ependymal cells rarely divide in the intact spinal cord (Tu et al. 2010; Stenudd et al. 2015). After spinal cord injury, ependymal cells rapidly divide and generate more than half of the astrocytes in the glial scar and a small amount of oligodendrocytes that myelinate axons (Tu et al. 2011; Stenudd et al. 2015). Oligodendrocyte progenitor cells are the main dividing cell population in the intact adult spinal cord. After spinal cord injury, they increase their rate of division and differentiate into remyelinating oligodendrocytes (Stenudd et al. 2015; Tu et al. 2010). Astrocytes sporadically divide in the intact adult spinal cord to maintain their population. After an injury, astrocytes become reactive, rapidly divide, and form the border of the glial scar (Fig. 8.4) (Tu et al. 2011; Stenudd et al. 2015). The border of the glial scar prevents the enlargement of the lesion (Tu et al. 2010, 2011). The beneficial function of the scar was further confirmed in a knockout mouse model of all *Ras* genes (Sabelstrom et al. 2013). The *Ras* gene knockout rendered the endogenous neural stem cells unable to proliferate, and consequently, the neural stem cell–derived component of the glial scar was never formed. When the proliferation of neural stem cells was blocked large cysts developed at the lesions while no cyst formation occurred in mice with normal neural stem cell function (Sabelstrom et al. 2013). This outcome implies that neural stem cell progeny functions as a scaffold within the scar to restrict secondary enlargement of the lesion and prevents the lesion from expanding after the primary injury. Thus, the neural stem cell-derived scar component restricts tissue damage and neural loss after spinal cord injury. The characterization of the distribution and phenotype of progeny (Sabelstrom et al. 2013; Tu et al. 2010, 2011), along with the quantitative contributions of each progenitor type to newly formed cells provide valuable insight into the endogenous cell replacement response to spinal cord injury, paving the way for advances in modulating specific populations of progenitor cells with the goal of promoting structural and functional recovery after spinal cord injury.

After spinal cord injury, neural stem cells migrate towards the lesion sites and integrate into the neuronal network (Tu et al. 2010, 2011). However, the potential success of stimulating endogenous neural precursor cells is hinged on delivery of various growth factors. This is the most common way to stimulate neural precursor

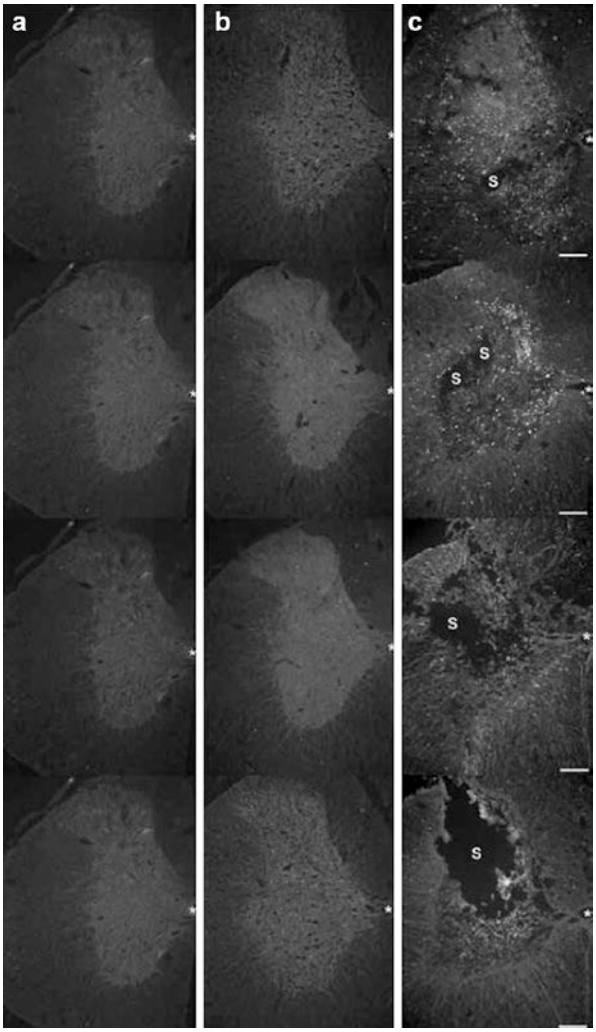

Fig. 8.4 A temporal profile of the secondary response to the primary spinal cord injury by proliferating cells and forming the border of the glial scar surrounding the focal lesion. Proliferating cells defined with Ki 67 immunoreactivity in the spinal cord of control rats and rats with spinal cord injuries. Ki 67+ cells (*white dots*) were rare in the gray matter with a few in the white matter in the spinal cord of intact (**a**) and sham-operated (**b**) controls. Animals with traumatic spinal cord injury demonstrated more Ki 67+ cells (**c**); these cells were predominantly located in the gray matter and form the border of the glial scar surrounding the focal lesion. Rows from top to bottom were 7, 14, 28, and 56 days after the traumatic spinal cord injury. This temporal profile of Ki 67+ cells demonstrated the secondary response to the primary spinal cord injury by increasing the number of Ki 67+ cells in the gray matter and forms the border of the glial scar surrounding the focal lesion. An a*sterisk* indicates the central canal. *S* spinal cord cavity. Bar = 200 μm (Tu et al. 2011) (Permission to reproduce the figure is granted to J.T. by the copyright owner, American Association of Neurological Surgeons (AANS), the publisher of *Journal of Neurosurgery: Spine*)

cells. The following growth factors are needed to stimulate neural precursor cells: epidermal growth factor, fibroblast growth factor-2 (Kojima and Tator 2000, 2002), basic fibroblast growth factor (Rabchevsky et al. 2000), acidic fibroblast growth factor (Lee et al. 2004), brain-derived neurotrophic factor (Namiki et al. 2000; Wang et al. 2013), vascular endothelial growth factor (Sharma 2003), nerve growth factor, neurotrophin-3 (Lee et al. 2004; Widenfalk et al. 2003), glial cell line-derived neurotrophic factor (Iannotti et al. 2004), insulin-like growth factor-1 (Sharma 2003), and stromal cell-derived factor-1 alpha (Imitola et al. 2004). They were reported not only to enhance the proliferation, migration and gliogenesis of neural precursor cells (Kojima and Tator 2000, 2002; Imitola et al. 2004) but also to protect the spinal cord from further damage (Sharma 2003; Widenfalk et al. 2003). In addition, these growth factors facilitate the regrowth of axons and remyelination (Lee et al. 2004; Namiki et al. 2000; Gensert and Goldman 1997). Functional recovery has been reported after growth factors were delivered into the injured spinal cord (Kojima and Tator 2000, 2002; Lee et al. 2004). However, the mechanisms of functionary recovery of the injured spinal cord by stimulating endogenous neural precursor cells are not fully understood. Neural stem cell progeny is necessary for the production of several neurotrophic factors that support neuronal survival after the primary injury. The loss of neurons is attributed to the loss of neurotrophic support from neural stem cell progeny (Sabelstrom et al. 2013). Therefore, stimulation of endogenous neural stem cells could be a potential therapeutic strategy for treatment of spinal cord injury.

8.5 Clinical Translation of Stem Cell Therapy in Spinal Cord Injury

The main purpose of state-of-the-scientific studies is to translate our discoveries into daily clinical practice. The basic research laboratory takes its observations obtained at molecular or cellular levels in a cutting edge state and implements this into acceptable clinical practice to the benefit of the public. However, this is always met with a lot of challenges, such as ethics, governmental regulations, funding constraints, the paucity of adequate collaboration among clinical and basic scientists, and the challenges while conducting clinical trials. From the identified gaps in the current state of the stem cell science and inherent challenges faced by the field, the author proposes six point schema for improving bench to bedside translation of stem cell therapy in Fig. 8.5a involving a rigorous network of six stakeholders: basic researchers, pharmaceutical companies, patients or general public participating in clinical trials, regulatory bodies or government agencies for providing research grant approval, collaborative research between basic and clinical scientists with the plan of developing biomarkers for potential drug targets and creating a concerted network of groups that identifies some of the medical problems relating to traumatic spinal cord injury. Patients with moderate traumatic spinal cord injury who suffer long-term complications are a major unmet medical need. Within our capabilities to

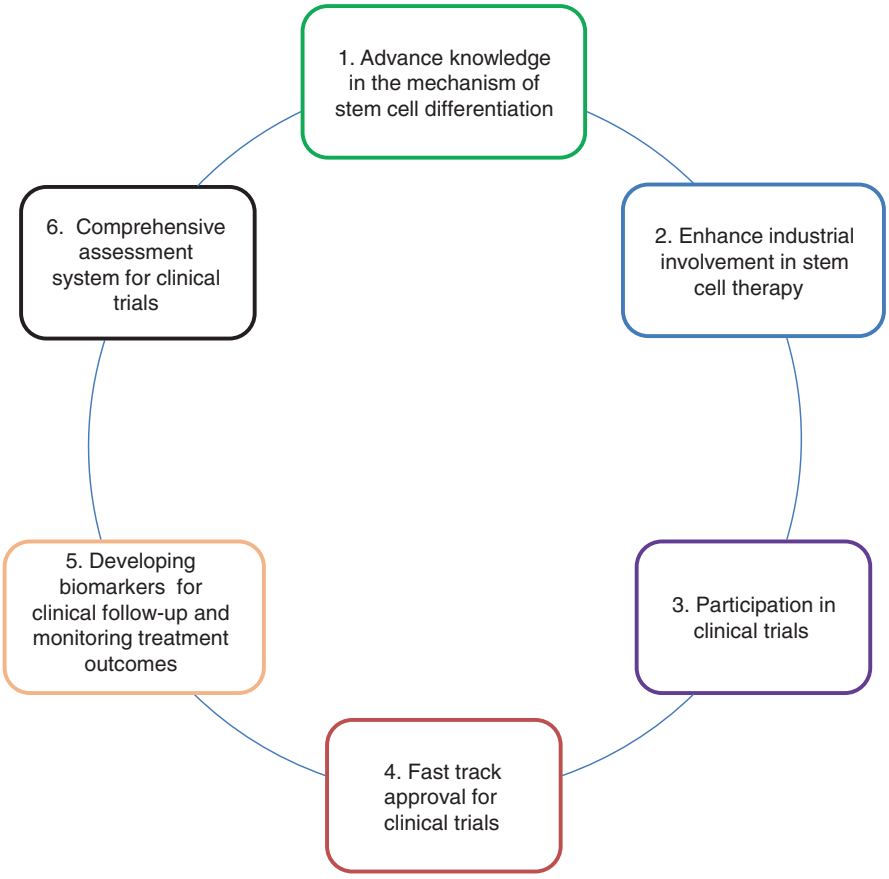

Fig. 8.5 (**a**) Proposed schema for effective translation involving the concerted effort of multilevel strategies of six main stakeholders.

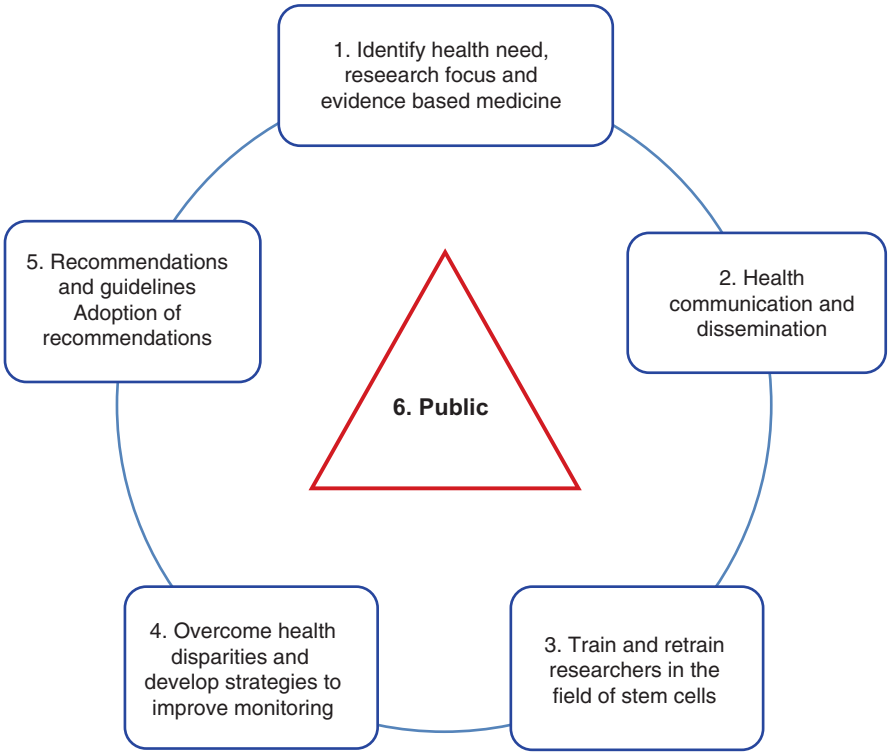

Fig. 8.5 (continued) (**b**) Proposed framework for the reinforcement of multilevel strategies effective clinical translation of stem cell therapy in spinal cord injury

clinically assess improvement, historically, the majority of individuals with moderate traumatic spinal cord injury are likely to recover to their pre-injury state. Early identification of those individuals likely to experience long-term complications is essential to maximize the benefit of stem cell therapy. Strategies to delineate this population from a larger population of individuals with moderate traumatic spinal cord injury could include enrollment of patients with persistent symptoms 1–2 weeks after injury, because recovery is most rapid in the first few days. Patients who are unlikely to fully recover could be identified using prognostic biomarkers including neuroimaging, biochemical, and objective clinical measures. Prognostic biomarkers are defined by the U.S. Food and Drug Administration as indicators that inform the natural history of a disorder in the absence of a therapeutic intervention (FDA 2014). Although identifying individuals with traumatic spinal cord injury who are most likely to respond to stem cell therapy and evaluating the biologic response to the therapy are essential for successful clinical trials, the ability to do either is lacking. Predictive biomarkers of stem cell therapeutic response are needed to address this challenge. Predictive biomarkers are baseline characteristics that identify individuals by their likelihood to respond to a stem cell therapy and may include biochemical markers including oxidative stress, inflammation, neuronal, axonal, and glial integrity, molecular imaging with positron emission tomography,

or functional imaging with functional magnetic resonance imaging. By identifying patients who are most likely to respond to stem cell therapy, the appropriate population can be selected for enrollment in clinical trials. Identifying specific predictive biomarkers would decrease the sample size needed to power clinical trials, thus decreasing risk to subjects, time to complete accrual, and cost. Biomarkers are dynamic measurements that show a biologic response occurred after stem cell therapy, including neuroimaging to measure effects on neuroprotection, neurorecovery, and neuroinflammation, or biochemical biomarkers of oxidative stress, inflammation, and neuronal integrity. Clinical trials would greatly benefit from biomarkers, which allow for the measurement of the effect of the stem cell therapy on the putative mechanism of a specific phenotype of stem cell's action, thus providing evidence of engagement of the target tissue by the therapy. To achieve stem cell repair, regeneration, and protection after traumatic spinal cord injury, each of the six points identified is critical for advancing the field, and efforts to address the points should be conducted in parallel to ensure ultimate success in improving clinical care and outcomes for individuals with traumatic spinal cord injury. We are still faced with the need to formulate hypothesis both at experimental and clinical epidemiologic level and to implement these into clinical practice while the translational researchers serve to collaborate and coordinate all these strategies to yield rapid results.

Indeed, communication and dissemination shown in Fig. 8.5b that is patient centredness will not only impact on the public, but will also help to tame the ethical issues in this field. Communication will involve both patients and clinicians involve in conducting randomized clinical trials. With strong feedback on outcomes, pharmacovigilance, and health promotion. Education of the populace in form of scientific advocacy is so paramount as this will impact on improved scientific collaboration, quality public control, and increased transparency among researchers and may improve funding of research work (Keramaris et al. 2008).

Research in neural stem cells is still a grey area, and much knowledge needs to be gained at the bench in order to actually close the knowledge gaps in stem cell therapy. There is inadequate understanding of the secondary spinal cord injury process after traumatic spinal cord injury, insufficient preclinical testing in diffuse axonal injury models, species differences, and lack of understanding of the mechanism of drug-receptor interactions. It has been suggested the need to use models for proper translation of stem cell therapy in traumatic spinal cord injury (Cao and Dong 2013). Academic and biotech researchers should address how to make their stem cell therapy products more feasible for commercial-scale production (Eaker et al. 2013). There is need for increased linkages and networking between academician, researchers, and clinician for the greater reward of what is being generated.

Methodological disparities between experimental models of traumatic spinal cord injury and clinical studies cannot be overemphasized. The intent to treat models, differences in statistical analysis as a result of different sample size, and different behaviours between human and animals. Animal research is a rapid, well-controlled, and cost-effective means to initially verify the hypothesis.

However, limitations exist in animal models of traumatic spinal cord injury and their application in stem cell therapy. First, because no single animal model accurately mimics all of the features of human traumatic spinal cord injury, individual investigators have appropriately refined experimental approaches to better fit their specific research goals. However, the resulting variability in experimental approaches among studies makes a comparison of results across laboratories and models difficult, limiting the confidence that results can be translated into successful clinical trials. Advancing preclinical research in animal models requires that results are comparable across studies and can translate into human studies. This requires standardization of available animal models and introduction of new models when scientifically necessary. Second, some of the popular current models do not correspond well with the human condition. Injury severities in animals differ from humans; while they are well defined in animals, it could take any direction in human. Third, preclinical studies should use the same level of rigor required for clinical trials. Specifically, assignment of animals to treatment conditions should be randomized, assessments must be conducted by blinded examiners, the primary outcome measure must be pre-determined, and statistical assessment of secondary outcome measures should utilize appropriate corrections for multiple comparisons. Fourth, the transplantation of stem cells into animal models should mimic the timing, delivery route, and the equivalent mass of cells feasible in humans. Last, the neurobehavioral outcome measures most widely used in preclinical models are not sufficiently sensitive to long-term functional deficits, and more sensitive rodent functional tasks that discriminate injury severity beyond 12 weeks after injury are needed. The need to improve study quality score has recently being called for by neurosurgical therapy academic industry roundtable, which was recently updated and this include the following recommendations: (1) Elimination of randomizations and assessment bias, (2) Use of a priori definitions of inclusion/exclusion criteria, (3) inclusion of appropriate power and sample size calculations, (4) full disclosure of potential conflict of interests, (5) evaluation of therapies in male and female animals across the spectrum of ages, and with comorbid conditions, such as hypertension and/or diabetes. Furthermore, some researchers have expanded on these proposed recommendations for improved clinical trials in spinal cord injury with a special focus on neuroprotective therapies in traumatic spinal cord injury (Cao and Dong 2013). Nonadherence was the single most important determinant of trial failure in the past.

Finally, the International Mission on Prognosis and Clinical Trial Design in traumatic spinal cord injury proposed ways of overcoming the above disparities and challenges. The recommendations include robust inclusion criteria and recommendations for general research in traumatic spinal cord injury (Cao and Dong 2013). The six-point schema is an overview recommendation with the public, patient or the society as the core and the fulcrum of all activities of research and if implemented may yield quality research outcome in neural stem cells therapy in spinal cord injury (Ugoya and Tu 2012).

8.6 Conclusion

Long-term disability from spinal cord injury is projected to rise globally. Neural stem cell therapy is a strategy that offers hope for the future in the treatment of spinal cord injury. In addition, we are now able to monitor autologous neural stem cells *in vivo*, cell migration, and clearly demonstrate that neural stem cells could selectively target injured spinal cord tissue and undergo neurogenesis. Finally, the proposed six points cyclical schema should be implemented with the determined effort of all stakeholders for effective clinical translation of neural stem cell therapy in spinal cord injury.

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Chapter 9
Stem Cell Clinical Trials for Multiple Sclerosis: The Past, Present and Future

Fakher Rahim and Babak Arjmand

9.1 Introduction

9.1.1 Background

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) that is associated by tissue inflammation and nerve cells apoptosis (Cudrici et al. 2006). The prevalence of this disease in women is 2 to 3 times more than men, and it is more frequent in the 20–40 years of age (Rolak 2003). The most important symptoms of MS include motor paralysis, sensory problem such as impaired sensitivity in one or more limbs and visual impairment, impairment of specific cognitive functions (McQualter and Bernard 2007). Identification of endogenous neural stem cells in the humans and rodents CNS has led to development new strategies for repairing the brain damages (Sun 2014). During development, the nervous system emerges from neural stem cell (NSCs) that have self-renewal potential and differentiate into neural and glial cell (Picard-Riera et al. 2002; Kriegstein and Alvarez-Buylla 2009; Vishwakarma et al. 2014). Two types of stem cells populating in patients with MS; one is hematopoietic stem cell (HSC) and others are mesenchymal stem cells (MSC) (Muraro and Uccelli 2010). Researches were carried out on 500 patients with MS worldwide that were treated with HSC since 1997 showed that disease progression stopped in many cases (Atkins and Freedman 2013). The second type is MSC, which have the ability to

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differentiate into various types of cells, and inhibit the cellular immune response against the nervous system (Krampera et al. 2007).

9.1.2 History

The word stem cells first appeared in 1908 in the scientific community. During an international conference in Berlin, a Russian expert in histology named Alexander Maximow for the first time used the term "stem cell" to describe his hypothesis of the existence of hematopoietic cells in the body (DiDio 1986). Later in 1924, he identified a certain type of cells among mesenchymal cells that have the ability to transform into various types of blood cells; thus, the first type of stem cells discovered and called "mesenchymal stem cells". In 1956, the first successful bone marrow transplant in the world was performed in a patient with leukemia by Thompson in New York. The patient was treated with radiation therapy followed by bone marrow (Bongso and Richards 2004). Later in 1990 the Nobel Prize in Medicine was awarded to Thompson for contributions to advance stem cell science (Bongso and Richards 2004). In the 1960s, Joseph Altman and Gopal Das discovered nerves cells production in the brains of adult human, which is also known neural stem cells (Altman and Das 1964). This finding was contrary to a longstanding belief which declared that in children after growth, new nerve cells will not generated in the brain and nervous system. In 1963, McCulloch and Till reported for the first time the production of stem cells in the bone marrow of mice (McCulloch and Till 1960). Many continuous efforts of scientists have been done to better understand stem cells and also trained numerous students who later became prominent researchers in the field of stem cell. In 1968, the first successful bone marrow transplant carried out by Robert A. Good for non-cancerous diseases (Day-Good and Peterson 2008). Human hematopoietic stem cells in the spinal cord discovered in 1978 (Prindull et al. 1978). In 1997, John's dick for first time identified tumor-initiating cancer stem cells (Werner et al. 2016). In 1998, James Thompson and colleagues were able for the first time to derive human embryonic stem cells (Thomson et al. 1998). In 2001, researchers simulated the early stages of human embryogenesis. In 2003, it has been shown dental pulp stem cells (DPSCs) can be extracted as a source of adult stem cells (Tavian et al. 2010). In 2005, researchers were able to restore a part of mobility to paralyzed mice by neural stem cells (Pomp et al. 2005). In 2006, scientists identified pluripotent stem cells from the spinal cord (Schroeder et al. 2016). The Nobel Prize in Physiology or Medicine 2007 has given jointly to Mario Capecchi, Martin Evans, Oliver Smithies, because of the valuable research in the field of embryonic stem cells (Jaryal 2007). In 2008, scientists reported the reproduction of the human knee cartilage with the use of adult mesenchymal cells (Huang et al. 2008).

9.1.3 Motivation

Multiple sclerosis (MS) is a chronic disease that affects the nervous system, which patients experience progressive deterioration of myelin. There is currently no cure for MS, but it is possible to treat the symptoms and reduce the number of relapses using various treatment modalities. Those suffering from MS are currently treated with drugs and, when possible, new treatment modalities such as myelin peptides (protein fragments) therapy. Because stem cells are part of the body's normal repair system, it represents an exciting new form of treatment for MS. Stem cell therapy might be used to develop new treatments for MS through preventing damage via resetting immune system known as immunomodulation, repairing the damaged myelin sheath known as remyelinating, and developing new medicines through growing nerve cells in the laboratory.

9.2 Stem Cell Therapy for MS

MS is one of the most common suggestions for stem cell therapy trials. Drug therapy could have a good response in some patients and may lead to slowing the relapses rate in primary course of disease, but would be unusable in progressive neurodegenerative phase. Most of patients with high relapses rate will develop secondary progressing form. So, the key concerns for stem cell therapy in the field of MS, including halting secondary progressing form, reverting disease and prevent progression in patients with high relapses rate, guarantee cessation of drug therapy and provide good quality of life, and offer the best alternative when all drugs are failing in patients with high relapses rate. The nervous system is a complex organ made up of nerve and glial cells, which surround and support neurons. Using stem cells could help to minimize and repair damage that has occurred in the neuron myelin (Fig. 9.1).

From the historical perspective, first attempt to use cell therapy for MS has been conducted more than 15 years ago. Since then, a lot of researchers have begun cell therapy trials around the world. In 2013, Ardeshiry lajimi et al, systematically reviewed the substantial clinical trials regarding the use of these stem cells and possible mechanisms in the treatment of MS (Ardeshiry Lajimi et al. 2013). They compared all available studies using various types of stem cells in MS, and reported that their findings may pave the road for the utilization of stem cells therapy for MS. Since first attempted to use stem cell therapy for MS, a lot of trials were started around the world of which overview some recent published studies have been mentioned in this review (Table 9.1).

Through searching all available clinical trials registries, since 2001 we found 49 records comprising 1605 patients with MS (Estimated No. of Patients), of which

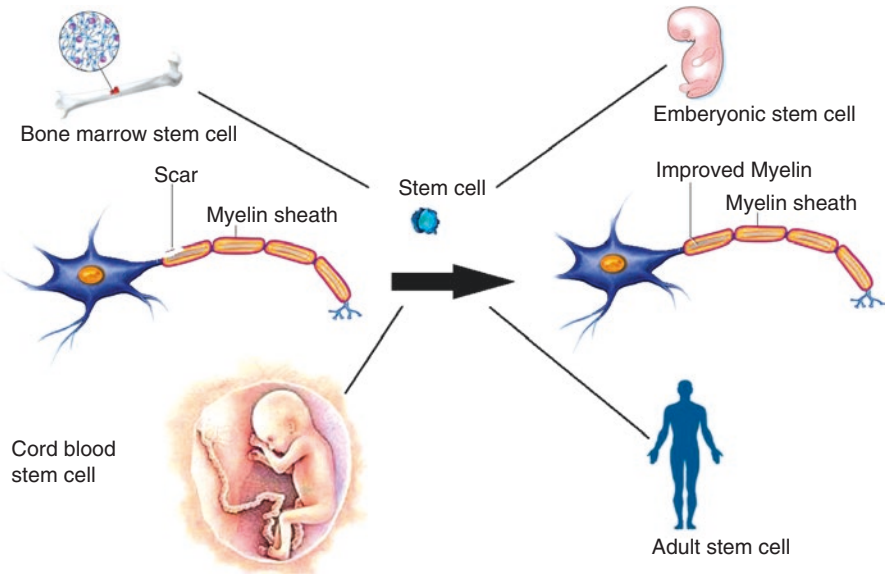

Fig. 9.1 Various types of stem cell that helps repairing the damaged myelin

only 7 were completed (Table 9.2). Most of the trials were either not started or terminated. Most of the trials were in phase I or in phase I and II. Phase I, include small number of patients, usually 20–100, with short duration (several months) to assess safety or dosage of particular pharmacological or non-pharmacological agent. MSCs, bone marrow or adipose derived, are currently being tested in more than 50 clinical trials around the world to treat MS, while HSC has been used in more than 20 trials so far.

9.3 The Potential of Hematopoietic Stem Cell (HSC) for MS

In 1999, Bielekova et al studied the mechanisms of action of autologous hematopoietic stem cell (HSC) in MS and reported clinical feasibility and relatively safe procedure and the induction of immediate immunomodulatory effects by this type of stem cells (Bielekova et al. 1999). In 2010, Novik et al conducted a trial on patients with MS using HSC and claimed that this type of stem cells show a real possibility to achieve a relapse- and progression- free period (Novik et al. 2010). In 2012, Bowen et al performed a trial using HSC for longer follow-up period (48-month) in patients with MS and reported that these stem cells are likely to be more effective in patients with less advanced relapsing/remitting MS (Bowen et al. 2012). In 2012, also Sepulveda et al conducted a clinical trial on small number of patients with MS during shorter follow-up period and stated that at short

Table 9.1 Available literature about stem cell therapy in multiple sclerosis (MS)

Author, year, country	No. of cases		Follow-up	Stem cell type	Clinical trial identifier	Outcome
	Stem cell	Placebo				
Mancardi et al. (2015) Belgium	11	10	6-month	AHSCT	EUCTR 2007-000064-24	strongly support further phase III studies with primary clinical endpoints
Nash et al. (2015) USA	25	–	18-month	AHSCT	NCT00288626	improvements in neurologic function
Rice et al. (2015) UK	40	40	12-month	BMD-MSCs	NCT01815632	safe and may reduce inflammatory parameters
Llufriu et al. (2014) Spain	5	4	12-month	BMD-MSCs	NCT01228266	Bone-marrow-MSCs are safe and may reduce inflammatory parameters
Li et al. (2014) China	13	10	12-month	UC-MSC	–	suggested a strong immunomodulation effect in MS patients
Lublin et al. (2014) USA	12	4	12-month	MLC[a]	–	safe and well tolerated in relapsing-remitting and secondary progressive MS
Burman et al. (2013) Sweden	12	9	12-month	AHSCT	NCT00273364	long-term disease remission seen after HSCT
S.M. N (2013) Iran	15	15	6-month	BMD-MSCs	–	safe without any adverse effect
Sepulveda et al. (2012) Spain	4	4	12-month	AMSCT	EUCTR2009-016442-74	at short time is safe and well tolerated
Connick et al. (2012) UK	5	5	10-month	BMD-MSCs	NCT00395200	suggestive of neuroprotection

(continued)

Table 9.1 (continued)

Author, year, country	No. of cases		Follow-up	Stem cell type	Clinical trial identifier	Outcome
	Stem cell	Placebo				
Bowen et al. (2012) USA	26	–	48-month	AMSCT	NCT00014755	more effective in patients with less advanced relapsing/ remitting MS
Karussis et al. (2010) Israel	8	7	25-month	BMD-MSCs	NCT00781872	Clinical feasibility and relatively safe procedure and induces immediate immunomodulatory effects
Novik et al. (2010) Russia	55	122	12-month	AHSCT	–	show a real possibility to achieve a relapse- and progression – free period
Bielekova et al. (1999) USA	16	9	6-month	AHSCT	NCT00342134	Clinical feasibility and relatively safe procedure and induces immediate immunomodulatory effects

hUC-MSC umbilical cord-derived mesenchymal stem cell, *BMD-MSCs* bone-marrow-derived- mesenchymal stem cell, *AHSCT a*utologous hematopoietic stem cells transplantation, *PDA-001* human placenta-derived cells, *AMSCT* autologous mesenchymal stem cell transplantation
[a]Mesenchymal-like cells (MLC) derived from healthy subjects (PDA-001)

time these stem cells are safe and well tolerated (Sepulveda et al. 2012). In 2013, Burman et al proposed a randomized study, HSC in patients with inflammatory/ relapsing MS despite treatment with alternate approved therapy (Burman et al. 2013). They showed a long-term disease remission seen after HSC transplant. Recently, Nash et al performed a trial study to determine the effectiveness of HSC as a new treatment for MS, a serious disease, in which the immune system attacks the brain and spinal cord, and reported improvements in neurologic function following HSC transplantation (Nash et al. 2015). In 2015, Mancardi et al conducted a multicenter, phase II, randomized trial including patients with secondary progressive or relapsing-remitting MS, to assess the effect of intense immunosuppression followed by HSC transplantation (Mancardi et al. 2015). They reported intense immunosuppression followed by HSC Transplantation is significantly superior to conventional drugs in reducing MRI activity in severe cases of MS. Over years or decades, HSC transplant for MS moved from myeloablative that basically killed some patients due to conditioning-related toxicities to non-ablative settings.

Table 9.2 Registered clinical trials using various types of human stem cells for the treatment of multiple sclerosis (MS)

Status	Main ID	Estimated No. of Patients	Stem cell type	Comparator	Publication	Route of administration	Phase	Date of registration
Completed	NCT01377870[a]	22	AMSC	cell free media	UP	IV	I and II	2011
Recruiting	NCT00273364	110	HSC	Standard treatment with a conventional drug	P	IV	III	2006
Not recruiting	NCT02034188	20	AMSC	–	UP	IV	I and II	2014
Not recruiting	NCT00497952	3	HSC	–	UP	IV	I and II	2007
Completed	NCT00395200	10	AMSC	–	P	IV	I and II	2006
Recruiting	NCT01730547	15	AMSC	–	UP	IV	I and II	2012
Not recruiting	NCT01606215	18	AMSC	Placebo	UP	IV	I and II	2012
Completed	NCT01895439	13	AMSC	–	UP	IV	I and II	2013
Unknown	NCT01099930	24	HSC	Standard Therapy	UP	IV	II	2010
Recruiting	NCT02403947	12	AMSC	Suspension media	UP	IV	I and II	2015
Unknown	NCT01854957	20	AMSC	Suspension media	UP	IV	I and II	2013

(continued)

Table 9.2 (continued)

Status	Main ID	Estimated No. of Patients	Stem cell type	Comparator	Publication	Route of administration	Phase	Date of registration
Unknown	NCT01364246	20	AMSC	–	UP	IV	I and II	2011
Not recruiting	NCT02035514	8	AMSC	Placebo	UP	IV	I and II	2013
Terminated	NCT00278655	21	HSC	–	UP	IV	I and II	2006
Completed	NCT01056471	30	AMSC	No Intervention: Placebo Control	UP	IV	I and II	2010
Enrolling	NCT02674217	200	HSC	–	UP	–	I	2015
Unknown	NCT00781872	20	AMSC	–	P	IV	I and II	2008
Terminated	NCT01228266	9	AMSC	suspension media	P	IV	II	2010
Recruiting	NCT02166021	36	AMSC	IV vs. IT	UP	IV and IT	II	2014
Completed	NCT00288626	25	HSC	–	P	IV	II	2006
Completed	NCT00342134	34	HSC	–	P	IV	II	2006
Completed	NCT00813969	24	AMSC	–	UP	IV	I	2008
Not recruiting	NCT01933802	20	AMSC	–	UP	IT	I	2013
Recruiting	NCT01883661	15	BM-MNC	–	UP	IV	I and II	2013
Unknown	NCT00927108	10	NPSC	–	UP	-----	II	2009
Recruiting	NCT02326935	100	AD-MSC	–	UP	IV	I	2014
Recruiting	NCT02239393	40	MSC	suspension media	UP	IV	II	2014
Completed	NCT00017628	20	PBSC	–	UP	IV	I	2001

Status	Main ID	Estimated No. of Patients	Stem cell type	Comparator	Publication	Route of administration	Phase	Date of registration
Completed	NCT00014755	35	PBSC	–	UP	IV	I	2001
Recruiting	NCT02587715	69	UC-MSC	Liberation therapy	UP	IT	I and II	2015
Terminated	NCT02418325	69	UC-MSC	Liberation therapy	UP	IT	I and II	2015
Recruiting	NCT02587806	69	BM-MNC	Liberation therapy	UP	IV	I and II	2015
Terminated	NCT02418351	69	BM-MNC	Liberation therapy	UP	IV	I and II	2015
No longer available	NCT02084121	–	HSC	–	UP	–	–	2014
Recruiting	NCT02495766	8	BM-MSC	Placebo	UP	IV	I and II	2015
Terminated	NCT01679041	1	HSC	–	UP	IV	II	2012
Recruiting	NCT01932593	6	BM-SC	–	UP	IV	I	2013
Recruiting	NCT01745783	30	BM-MSC	Placebo: Lactated Ringer's solution, 2.5% glucose and 1% human albumin	UP	IV	I and II	2012
Recruiting	NCT02157064	100	ASC	–	UP	–	–	2014
Not recruiting	NCT01453764	10	ASC	–	UP	IV and IT	I and II	2011
Recruiting	NCT01815632	80	BM-SC	–	P	IV	II	2013

(continued)

Table 9.2 (continued)

Status	Main ID	Estimated No. of Patients	Stem cell type	Comparator	Publication	Route of administration	Phase	Date of registration
Completed	NCT00040482	10	HSC	–	UP	IV	II	2002
Not recruiting	ACTRN12616000151437[b]	20	HSC	–	UP	IV	II	2016
Not recruiting	ACTRN12615000687594	45	ASC	–	UP	IV	I	2016
Not recruiting	CTRI/2014/11/005231[c]	15	MSC	standard drug regime	UP	IV	I and II	2014
Not recruiting	ACTRN12613000339752	30	HSC	–	UP	IV	II	2013
Ongoing	EUCTR2010-023560-40-SE[d]	20	HSC	standard drug regime	UP	IV	II	2011
Terminated	EUCTR2007-000586-38-IT	8	MSC	Other drug (dell'autotra)	UP	IV	II	2008
Ongoing	EUCTR2006-002562-19-IT	12	HSC	–	UP	IV	II	2007

AMSC autologous mesenchymal stem cell, *HSC* hematopoietic stem cell; *IV* Intravenous injection, *IT* intrathecally injection, *BM-MNC* autologous bone narrow derived mono nuclear stem cell, *NPSC* neuronal progenitor stem cell, *AD-MSC* autologous adipose derived mesenchymal cell, *PBSC* autologous peripheral blood stem cell, *UC-MSC* allogeneic human umbilical cord tissue-derived mesenchymal stem cells, *BM-MSC* autologous adult bone-marrow mesenchymal stem cells, *BM-SC* autologous adult bone-marrow stem cells, *ASC* adipose stem cell (), *P* published, *UP* unpublished

[a]A service of the U.S. National Institutes of Health (NCT), Website: www.clinicaltrials.gov

[b]Australian New Zealand Clinical Trial Registry (ACTRN), website: www.anzctr.org.au

[c]Clinical Trials Registry-India (CTRI), website: www.ctri.nic.in

[d]EU Clinical Trials Register (EUCTR), website: www.clinicaltrialsregister.eu

9.4 The Potential of Mesenchymal Stem Cell (MSC) in MS

MSC, derived from adipose tissue or bone marrow were anticipated as an alternative to HSC transplant in MS. The proposed mechanism of action is potentially suppression of auto-reactive T-cells called immunomodulation. Sever immunosuppression is not necessary in case of MSC therapy, because immune system is not going to be destroyed, as well as the therapy suppose to be not toxic at all. In 2010, Karussis et al propose an explorative trial with the both intrathecally and intravenously injection of MSC in patients with MS, to prevent further neurodegeneration through neuroprotective mechanisms and restoration of neuronal function (Karussis et al. 2010). They reported clinical feasibility, relatively safe procedure and induces immediate immunomodulatory effects in patients with MS. In 2012, Connick et al conducted a trial to assess whether intravenous injection of bone marrow-derived MSC is a safe novel therapeutic approach for patients with MS or not, and suggest a neuroprotection effect (Connick et al. 2012). In 2013, Nabavi et al claimed that MSC is safe without any adverse effect in patients with MS (S.M. N 2013). In 2014, Llufriu et al conducted a randomized Phase II study, masked and crossed-over with placebo to evaluate the safety and tolerability of MSC transplantation in patients with active MS (Llufriu et al. 2014). They reported bone-marrow-MSCs are safe and may reduce inflammatory parameters. Finally, in 2015, Rice et al conducted a prospective, randomized, double-blind, placebo-controlled trial using bone-marrow-MSCs in patients with progressive MS and showed that it is safely improve conduction in multiple central nervous system pathways affected in these patients (Rice et al. 2015).

9.5 The Future of Stem Cell Therapy

Despite major knowledge advances since the discovery of stem cells, the field is so broad and there are still many valuable opportunities to work. Every year, many people suffer from their internal organs damages that lead to life-threatening complications or losing their lives. Laboratory production of new tissue that could potentially be transplanted into the patient's body can be a solution to this big problem.

9.6 Conclusion

Prior to implementation of future investigator-initiated clinical trials, a realization of stem cells therapy or transplantation to demonstrate its feasibility to target the therapeutic area without any adverse effects is necessary. Some pre-clinical trials in animal models provide a robust evidence to advise that this safe and

feasible therapeutic approach has a protective effect on motor neuron degeneration and potentially enhance function in MS patients. But it should be considered that these approaches are subject to statute of limitations such that final confirmation in humans with MS. Actually, considering the safety of current new stem cell transplantation approaches for MS, this chapter may highlights that it is the time to move into the clinic to really appreciate whether they work in these patients or not.

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Chapter 10
Stem Cell Trials for Retinal Disease: An Update

Henry Klassen

10.1 Introduction

Within the nascent field of regenerative medicine, it is striking that applications to the retina have received a relatively large share of attention. This can be attributed to the convergence of a number of factors, including the relative accessibility of the target tissue, the sophistication of retinal diagnostics and interventions, the underlying progress in neural progenitor and RPE cell research, the safety factor manifest by patients having two eyes, and of course the large unmet medical need. Perhaps less agreed upon, but potentially significant, is the status of the retina as an immunologically privileged site. Taken together, these factors have propelled this line of therapeutic research from laboratories into clinical trials. The projects vary in terms of disease target, cell of interest, and method of delivery, as has been discussed previously (Klassen 2015; Zarbin 2016). Here I would like to revisit this topic and update the status of these efforts.

10.2 Cell of Interest

A key aspect of any cell-based therapy is obviously the type of cell to be used. In this regard, I find it helpful conceptually to divide these into CNS derivatives versus other sources ectopic to the retina and CNS.

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The CNS cell types currently in use comprise retinal pigment epithelial (RPE) cells and neural progenitors, either of forebrain or retinal origin. The RPE cells are in turn generally derived in the laboratory from pluripotent stem cell lines, either embryonic (ES) or induced pluripotent (iPS) cells. The brain-derived cells are variously referred to as neural stem cells (NSCs), neural precursor cells (NPCs), or brain progenitor cells (BPCs), while the retinal progenitor cells are generally referred to as RPCs. Cells from origins ectopic to the retina/CNS include the so-called mesenchymal stem cells (MSCs), hematopoietic stem and progenitor cells (HSPCs), as well as umbilical and adipose cell types.

10.2.1 RPE Cells

The retinal pigment epithelium is a monolayer of darkly pigmented cells lying deep to the retina. This structure is disrupted in a number of retinal conditions, most notably age-related macular degeneration (AMD). New RPE cells can be grown in the laboratory setting by differentiation of pluripotent stem cell types, and identified and isolated by virtue of their vivid pigmentation and propensity to grow as a confluent monolayer. The availability of stem cell-derived RPE cells, combined with unmet medical need in AMD, has resulted in the relative popularity of using the former as a treatment for the latter, evidenced by the multiple clinical trials employing this strategy that are currently underway.

10.2.2 CNS Progenitors

Cells of this type retain a proliferative capacity, but are not pluripotent and exhibit developmental restriction to the neural lineage, i.e., the generation of neurons and glia. As a result of this tissue restriction they are multipotent. Such progenitors are not immortal and tend to senesce with extensive passaging, which presents a challenge when contemplating large-scale production. On the other hand, the cells retain considerable plasticity, are pre-specified to differentiate into neural cell types, and do not require a pre-differentiation step prior to transplantation. This last characteristic greatly simplifies the manufacturing process. In terms of developmental potential, all CNS progenitors can give rise to neurons and glia, however, the specific subtypes of these cells generated are known to vary depending on tissue source.

10.2.3 Forebrain Progenitors

The forebrain is generated by the activity of neural precursor cells during development, but also retains small populations of neural stem cells in some locations into adulthood. All these cells are characteristically defined by their ability to generate neurons, astrocytes and oligodendrocytes.

10.2.4 Retinal Progenitors

The neural retina (as opposed to the RPE layer) is generated by the activity of retinal progenitor cells during development, however, there is a notable lack of retention of such activity once the retina has reached its final size. Unlike other neural progenitors, RPCs have the capacity to generate photoreceptor neurons, but do not give rise to oligodendrocytes.

10.2.5 Non-CNS Cells

The successful isolation and transplantation of CD34+ hematopoietic progenitor cells from bone marrow and cord blood has long represented the cutting edge of regenerative medicine in the clinical domain. The success of this technology has been associated with growing availability of clinical grade allogeneic cells of this type.

Clinicians in particular have also directed attention to the use of mesenchymal and, more recently, adipose-derived cells. Some of the enthusiasm for these cells may relate to the convenience of using autologous material, generated as needed, without the same degree of regulatory oversight incumbent on other experimental therapies. This may be expeditious from a certain standpoint, but there may also be less underlying research to support and guide the studies.

Work with the CNS cell types is technically demanding and these types of cells are not as readily available as human clinical product from existing sources or the patients themselves, the way bone marrow and adipose derivatives are. Accordingly, the projects utilizing CNS cell types have active research activities supporting the clinical efforts, whereas the other types of projects may outsource much of the product development component. There are evident advantages and disadvantages to either approach, from a practical standpoint alone. Personally, I find it easier to make a case for the use of local retinal cell types, but ultimately it will come down to demonstrated efficacy in patients and that has yet to be determined. The CNS cell-based trials may receive somewhat greater attention here, if only out of greater familiarity on my part.

10.3 Mechanism of Action

Another difference between therapeutic approaches revolves around whether the major objective is to replace a cell type lost to the disease process, or to rescue host neurons before they die.

The RPE-based projects clearly emphasize cell replacement as the goal, although these cells might also have a paracrine effect on photoreceptors, e.g., as mediated by secretion of PEDF. The CNS progenitor-based approaches can be directed towards cell replacement or neurotrophic activity, depending on additional considerations.

Being ectopic to the retina, and non-neural in origin, hematopoietic and mesenchymal stem cells seem unlikely candidates for integration into the neural circuitry of the eye. The mechanism of action for these cell types is not well defined, but might be neurotrophic or, perhaps more likely, immunomodulatory.

10.4 Disease Target

Age-related macular degeneration (AMD) is the most popular disease target among retinal diseases. This can be attributed to the prevalence of the condition as well as the relative ease of deriving RPE cells from pluripotent cultures. As mentioned above, AMD is a degenerative condition that disrupts the RPE layer and mechanistically this occurs prior to the loss of photoreceptors. AMD comes in two basic "flavors", wet and dry, referring to the neovascular and atrophic variants, respectively. The wet form can be further divided to include less common forms such as RPE detachment and RPE tears, both being associated with neovascular abnormalities.

Stargardt's disease is a rare, genetic maculopathy associated with multiple forms of inheritance. As such, it is a macular condition with RPE involvement that qualifies for orphan status.

Retinitis pigmentosa (RP) is also a rare, genetic condition, but with primary photoreceptor involvement. Characteristically, rods are lost first, followed by cones. RPE cells migrate into the retina once the outer nuclear layer is lost, leading to the pigmentary changes that give RP its name.

Other retinal conditions have also been targeted for cell-based trials, including myopic degeneration, vascular occlusive diseases, and optic nerve conditions.

10.5 Method of Delivery

The major distinction between trials in terms of delivery is between transplantation to the subretinal space and simple intravitreal injection. A further distinction is whether the cells are delivered as an intact layer or as a cell suspension. Yet another consideration is whether the cells can be thawed for immediate use or are maintained in culture just prior to injection.

Because of the recognized difficulty in achieving integration of mature cells into the RPE monolayer, RPE-based projects generally transplant the cell product as an intact epithelial layer, not infrequently adherent to an artificial scaffold to enhance engraftment. In this way the graft constitutes a defined therapeutic "patch" rather than the spontaneous remediation of a defect in the RPE monolayer by dissociated donor cells.

When CNS progenitors are used for cell replacement, the product would typically be placed in the subretinal space. The rationale is that donor cells can more

readily integrate into the adjacent retinal outer nuclear layer from that location. For neurotrophic activity, some groups deliver the product to the subretinal space, while others opt for the vitreous cavity.

For hematopoietic and mesenchymal cells, an extraretinal injection appears to be the route of choice for administration to the retina. This would generally imply an intravitreal approach, however, apparently some groups have attempted extraocular, e.g., retrobulbar, injection as well.

From a practical perspective, it is clearly preferable that a therapeutic cell product be readily available for administration with a minimum of preparation needed at the clinical site. This would tend to imply a frozen product that can be stored and thawed on short notice for clinical use. Despite the obvious benefit of such an approach, it may not always be expeditious or feasible with current technology. There is evidence to suggest that re-culturing cells for a period after thawing can have a significant positive impact on cellular function (François et al. 2012).

10.6 Update on Clinical Trials

World wide, there are at least a dozen different trials using stem or progenitor cells in the retina, at various stages of progress. Here I will look at CNS cell-based trials first, roughly in order of apparent current progress, followed by a consideration of other cell types.

10.6.1 Phase 2b/Efficacy

Two groups using CNS-type cells have now successfully completed safety trials and are therefore in a position to pursue clinical proof-of-concept. One of these is Ocata (formerly ACT), now owned by Astellas. The other is StemCells, Inc.

Ocata is testing an ES-derived RPE product in dry AMD, Stargardt's disease, and now myopic degeneration. The dissociated cells are injected subretinally as a single bolus. CHA Biotech has a license for use of this technology in South Korea and has completed a smaller trial in local patients with AMD and Stargardt's disease (Song et al. 2015). Ocata's Phase 2 proof-of-concept trial, PORTRAY, is listed as "recruiting" on the clinicaltrials.gov website and a follow-on trial from CHA Bio is also anticipated. Of note, Stargardt's disease is no longer listed as an indication.

StemCells, Inc. has completed their Phase 1/2a trial with human neural stem cells in dry AMD and initiated their follow-on RADIANT trial. However, the company recently announced that they would divert limited financial resources to support their spinal cord trial. The RADIANT trial is currently listed as having "suspended recruitment" on the clinicaltrials.gov website.

10.6.2 Phase 1/2a Dose Escalation

One group is now well into initial dose ranging studies and that is jCyte, of which I am a founder. jCyte uses allogeneic retinal progenitor cells which are injected into the vitreous cavity without immune suppression. The initial disease target is RP. At this time, dosing has been completed in the first of two patient cohorts. The doses tested were 0.5, 1, 2, and 3 million cells. Dosing in the second, better-seeing cohort is currently underway.

10.6.3 Phase 1

A number of other groups have successfully initiated safety trials in the retina and presumably will also progress through dose escalation studies. Groups that have initiated early trials include the RIKEN/Healios effort with iPS-derived RPE and both Cell Cure Neurosciences (a subsidiary of BioTime) and the London Project with ES-derived RPE, all in various forms of AMD.

The RIKEN trial was halted when, through extensive genetic screening, the autologous iPS product was found to have mutations that might theoretically increase tumorigenicity. No actual evidence of such behavior was evident. The current plan, as I understand it, is to switch to allogeneic iPS cells banked according to MHC background, which is somewhat less diverse among the Japanese population. Another effort with ES-derived RPE in dry AMD from Regenerative Patch Technologies is imminent, but dosing has yet to be announced as this is written. ReNeuron initiated their trial using retinal progenitor cells in RP earlier this year. They are pursuing a subretinal approach.

10.6.4 Non-CNS Cell Types

The University of Sao Paulo group completed both Phase 1 and 2 studies of autologous bone marrow stem cells in RP, delivered by intravitreal injection. The date of completion is listed as 2013. A safety study at Mahidol University in Thailand using intravitreal bone marrow-derived mesenchymal stem cells in RP is listed as “enrolling by invitation” and was due to finish in 2014.

A pilot safety study using autologous bone marrow-derived CD34+ hematopoietic stem cells was initiated at the University of California, Davis in 2012. The cells are injected intravitreally in RP, AMD, and a range of retinopathies. The study is listed as “enrolling by invitation” with estimated completion in 2017. Another study with intravitreal injection of autologous bone-marrow derived stem cells is being performed by Red de Terapia Celular in Murcia, Spain. This study includes a subconjunctival placebo control in the fellow eye and is due to be completed later this

year, according to entries on clinicaltrials.gov. Another trial using autologous bone marrow cells by Stem Cells Arabia is underway in Amman, Jordan, another at Chaitanya Hospital, Pune, India, and another in Florida and Dubai by MD Stem Cells.

In addition, Janssen is testing their umbilical tissue-derived cells subretinally in a Phase 1/2a study in dry AMD, with fellow eye controls. The study is listed as "ongoing but not recruiting participants." A prior study in RP was terminated.

10.7 Conclusion

There are upwards of a dozen active trials using stem cells for retinal diseases. The above list was intended to be current, but might not be entirely complete. At this stage the majority of these projects are still in initial safety and dose escalation trials. The most popular cells are pluripotent cell line-derived RPE and autologous bone marrow-derived cells. The former are all delivered to the subretinal space and the latter to the vitreous, based on presumed mechanism of action. Also popular are allogeneic CNS progenitors, delivered either under the retina or to the vitreous. Many of these trials have been active for several years, implying that safety concerns have been few, and this conclusion has been backed up in a number of published reports. Only two efforts are currently poised to directly investigate efficacy at this time, with one suspended due to funding limitations, so we will have to wait a while longer to get answers to the all-important question of whether these transplants will have an impact on their intended disease targets.

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Chapter 11
Stem Cells in the Management of Tympanic Membrane Perforation: An Update

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11.1 Introduction

The healing process of a perforated tympanic membrane (TM) occurs through many complex steps of cell proliferation and migration and the interplay of a series of biomolecules. Moreover, the management of severe tympanic membrane perforation attracted a good deal of attention especially where surgical intervention was considered as the number one choice. However, with the advancement in molecular and stem cell research new approaches are being considered and throughout the last decade, multiple publications dealt with regeneration of TMPs.

Some work appeared using biomolecules like PDGF (Platelet derived growth factors), Hyaluronic acid (HA), epidermal derived growth factor (EGF) and

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pentoxifylline. Other attempts used scaffolding materials like Calcium alginate, silk and Chitosan including the use of stem cells (Hong et al. 2013).

In this review, a 10 year search was performed through PubMed, Scopus, and Mednet and using key words like: tympanic membrane repair, perforations, and stem cells in the eardrum.

Anatomically, the tympanic membrane constitutes the medial boundary of the external acoustic meatus and the lateral wall of the middle ear. Histologically, it is composed of three layers, from outside to inside, the continuation of the skin inside the external ear canal (stratified squamous epithelium), a core of radially and circularly arranged collagen fibers (connective tissue) and the mucous membrane lining the middle ear (simple cuboidal epithelium) (Ross and Pawlina 2014).

It is well known that the major cause of tympanic membrane perforation is infection seconded by trauma. Traumatic tympanic membrane perforations usually occur due to a blow on the ear, skull base fracture, barotrauma due to severe atmospheric overpressure, exposure to excessive water pressure (e.g. in scuba divers), and iatrogenic causes. The common symptoms of a tympanic membrane perforation are usually purulent discharge through the ear, whistling sounds when blowing the nose, tinnitus and hearing loss.

A review of statistics related to the incidence of TM perforation in the general population does not provide exact and reliable numbers. However, analysis of the yearly surgical TM repairs performed in the USA shows that in a population of 280 million, approximately 150,000 tympanoplasties are performed (Howard 2014).

For this review the focus is on the role of stem cells and their related products in the management of tympanic membrane perforations, by searching for publications in the past 10 years using engines like PubMed, Medline and Scopus. The results of the search amount to a series of twenty four articles covering methods used for regeneration of TMPs including scaffolding materials, biological material like growth factors, or stem cells.

This review while taking into consideration the conventional methods of TMP repair, will highlight mostly advances in the use of biological materials including stem cells.

11.2 Management Modalities for Tympanic Membrane Perforations

Most tympanic membrane perforations tend to heal on their own. Usually, a healed TM perforation consists of a thin membrane, called neomembrane, made of squamous epithelial cell layers and a mucosa without a fibrous middle layer. It is usually very thin and can be sometimes mistaken under microscopy for a perforation.

These neomembranes can develop into a retraction pocket, especially when they are in the posterior superior quadrant of the drum and can predispose to cholesteatomas.

Throughout history, several surgical methods were developed to repair tympanic membrane perforations. The common procedures for repair of traumatic TMP's are paper patch repair, with a thin film of paper (example: cigarette paper), and tympanoplasty through reconstruction of the tympanic membrane surgically with temporal muscle fascia and tragal cartilage. These methods have been and still are the most common methods in use (Howard 2014).

11.3 Scaffolding Material

The recent methods used for regeneration of TMPs make use of scaffolding materials like Calcium alginate, silk and Chitosan.

11.3.1 Calcium Alginate

For the past 10 years, only one article was encountered whereby Calcium alginate showed promising results in the healing of TMPs. Alginate is an organic polymer derived from Seaweed with well-documented scaffolding capabilities optimal for cellular ingrowth. Moreover, it has been successfully used in the management of chronic wounds. Its mechanism of action consists in the formation of a calcium alginate compound which leads to the control of the physical properties of the scaffold. This property is due to the crosslinking between the alginate molecules to calcium, consequently, it increases the resistance and improves the handling of the product (Weber et al. 2006).

In this study, by Weber et al. in 2006, calcium alginate for TM grafts was developed using a computer aided design (CAD). The structure of the graft and its implantation technique were similar to a standard biflanged tympanostomy tube. Chronic TMPs were created in Chichilla models, approximately 5 mm in diameter using a thermal cautery. The ears were grouped into (1) a control without a patch, (2) another group with a paper patch, and the third group with calcium alginate plugs. The animals were sacrificed 10 weeks post implantation and the TMs were microscopically inspected. The calcium alginate treated TMPs showed a significantly improved healing rate over the other two groups: 9 out of 13 healed in the third group with alginate versus 2 of nine in the second paper patch group and 1out of 11 in the control. Auditory Brainstem Reaction (ABR) thresholds using Intelligent Hearing Systems, ABR systems, showed that the calcium alginate was not ototoxic (Weber et al. 2006).

11.3.2 Silk

Reports in the literature considered silk as a probable scaffolding material to be used in the regeneration of TMPs (Ghassemifar et al. 2010). This is a protein polymer with a high degree of elasticity. The silk fibroin is biocompatible and as documented through its use for long time, it helps also peptides to get bonded to enhance cell attachment (Sofia et al. 2001).

In 2010 Ghassemifar et al. showed that human tympanic membrane cells (hTMCs) obtained from pars tensa explants (2–3 mm in size) of patients were subjected to extensive skull-base surgery whereby the TM and related structures were sacrificed (Ghassemifar et al. 2010). These cells were cultured for 15 days in a serum containing medium then harvested, seeded on membranes prepared from silk fibroin (BMSF) and on tissue-culture plastic membranes. They were then stained using antibodies and immunofluorescent techniques. The direct cell counts indicated an excess of 70 % hTMCs cells growing on BMSF as compared to tissue-culture plastic membranes. This increase in cell number suggests that a BMSF membrane can provide a more suitable substratum for growth of hTMCs, compared to culture plastic membranes (Ghassemifar et al. 2010). As a conclusion, this result shows a promising future for silk fibroin in the construction of tissue engineered replacements for tympanic membranes or possibly others (Ghassemifar et al. 2010).

In two studies, Kim and coworkers in 2008 and 2010 compared 2 methods of repair of TMPs; the Silk patch vs. the paper patch. In this in vivo study, artificial bilateral 1.8 mm myringotomies were performed on 50 adult rats. The perforated TM's on the right ear of 40 rats were managed with silk patch while those on the left ear with paper patch. 10 rats were used as control. The silk material was derived from silkworm cocoons. In this study the mechanical properties were studied, followed by endoscopic observation and histological examination of the TM. The tensile strength of the silk fibroin patches were similar to that of the paper patch but the flexibility was 6 % much more compared to 0.2 % for paper patch. Silk fibroin patches were tougher and easier to use (Kim et al. 2010).

Daily endoscopic examination with photo-documentation was done on the 50 rats. It showed that the mean healing times were significantly different between the silk-patch-treated TMs and paper-patch-treated TMs, 7.2 ± 1.48 and 9.1 ± 1.11 days, with a mean difference of 1.9 days shorter in favor of the silk-patch treated ears. After 14 days, the proportion of completely healed ears in the silk patch group was 37/40 i.e. 93 %, compared to 28/40 i.e. 68 % in the second group with paper patch and 13/20 i.e. 65 % in the control group. The treated tympanic membranes were studied histologically at days 5, 7, 9 and 14. After 2 weeks, the TMs treated with silk patches had a normal contour and were twice as thick as those of day 9 (Kim et al. 2010). In brief, this group shows a faster and significantly better organized healing compared to paper patch and control groups.

11.3.3 Chitosan

Chitosan originates from Chitin, a predominant polysaccharide found in the exoskeleton of crustaceans. It enhances significantly the mitogenic activity and is widely used in tissue engineering studies (Kind et al. 1990; Muzzarelli et al. 1994; Badylak 2002; Chung and Burdick 2008). The first clinical trial, using autologous serum eardrop therapy with a chitin membrane (ASET) for closure of chronic TMPs without any surgical intervention was done by S. Kakehata et al., in 2008. This study included 19 patients with chronic TMPs. Before treatment with ASET, the TM perforations were cauterized chemically and covered only with a chitin membrane. The perforation sizes were grouped into: small (<1/4 of the TM), medium (1/4-1/2 of the TM), and large (>½ of the TM). The numbers of ears with small, medium and large perforations were 14, 1, and 4 respectively (Chung and Burdick 2008).

Various active factors were measured like the concentration of epidermal growth factor (EGF), transforming growth factor β 1(TGF β-1), fibronectin, and interleukin (IL) 6. The results showed that complete healing of the TMPs occurred in 11 (58 %) of 19 ears and reduction of the perforation size was observed in 2 ears (11 %). Closure rates for small, medium, and large perforations were 57, 0, and 75 %, respectively. The ASET treatment showed no remarkable side effects. In conclusion, ASET was considered as a very safe method that requires no surgical intervention. It is easy to use, suitable for office and home use, and is very efficient for the treatment of chronic TMPs.

In another study by Kim et al., in 2008, a biocompatible water-insoluble chitosan patch was developed. These water-insoluble patches were treated with acetic acid and glycerol to obtain the Chitosan Patch Scaffold (CPS) and were compared to the conventional paper patch. Several criteria were considered including thickness of the CPS and paper patch, mechanical properties, morphological analysis, water contact angle test, cytotoxicity and study for the proliferation of the TM cells (Kim et al. 2009). The morphological study showed that the CPS are more resistant to infection compared to others and are not toxic (Kim et al. 2009). Mechanically chitosan showed a significant tensile strength when 3 % glycerol was added to 3 % chitosan in comparison with the very nonflexible property of the paper patch which could easily detach from the TM (Kim et al. 2009). Analysis of water contact angle showed that the higher the glycerol concentration the more the hydrophilic property of the chitosan patch is. In rats, the transmission electron microscope examination showed that the cells of the TM proliferate faster under CPS guidance (Kim et al. 2009).

In conclusion, a new artificial TM patch composed of 3 % of chitosan and 3 % glycerol showed more effectivity than spontaneous healing of traumatic TMPs, with no cytotoxic effects. A sequel study done by the same team in 2013 used an EGF-Releasing chitosan patch to study the regeneration of TMPs. Knowing that EGF was reported to be very effective for the recovery of skin wounds by helping the migration

of fibroblasts, endothelial and then vascular cells, primary TM cells were harvested from the TMs of rats, then EGF-CPS were prepared as previously reported but with a different concentration of glycerol and chitosan. Results of this study showed that EGF-CPS had better mechanical properties and greater cell viability than established CPS. In addition they showed a much higher wound-healing rate than CPSs (Seonwoo et al. 2013).

11.3.3.1 Use of Biomolecules in the Management of Tympanic Membrane Repair

As mentioned before, the process of tympanic membrane healing is complex. It requires the orchestration of various elements including cell migration and proliferation, the presence of inflammatory cells and respective secretory products, in particular, during the first stages of the healing process. The addition of biomolecules, either in drops or via scaffold soaked with these molecules, may provide an enhancement to this healing process (Hong et al. 2013).

11.3.3.2 Basic Fibroblast Growth Factors (bFGF)

b-FGF gained a notable place in the large pool of biomolecules studies in regards to tympanic membrane perforation healing (Kanemaru et al. 2011), used gelatin sponges imbibed with either b-FGF or in saline. They randomized their application on 63 chronic tympanic membrane perforations; fibrin glue was added as a sealant. The b-FGF treatment proved to be significantly ($p<0.0001$) superior in regards to complete closure of the perforation as compared to the control; 98.1 % of the study group achieved complete perforation closure following four courses of treatment as opposed to 10 % for the control group. On evaluation 3 weeks post-treatment, symptoms such as tinnitus and aura fullness were also significantly improved in the study group ($p<0.0001$) as compared to the control. There was also noted absence of infections and major sequelae in treated patients which added to the effectiveness of b-FGF treatment (Kanemaru et al. 2011).

Similar outcomes were obtained in a study by (Zhang and Lou 2012). The study focused on the effect of b-FGF on tympanic membrane perforation due to direct traumatic penetration, however, only small sized perforations were included in this study. The study group, treated with daily direct application of b-FGF drops, showed, compared to control, a statistically significant 23 % improvement ($p=0.01$) in perforation closure rate and a threefold shortened perforation closure time ($p<0.01$). However, 3 months following treatment, audiometric assessment revealed failure of b-FGF treatment to give significant hearing improvement compared to the other groups. Lou in 2012 also used FGF to study its healing effect on TM however; his emphasis was on large traumatic eardrum perforations estimated at 50 % or more of the entire tympanic membrane. A total of 94 patients were split into 3 groups: (1) a control group (36 patients), (2) a group to which direct FGF

application was performed (36 patients), and (3) a group to which FGF was applied via Gelfoam (35 patients). Both FGF and FGF via Gelfoam groups showed a significant enhancement in the closure rate and closure time compared with the control group ($p < 0.05$) but did not show a significant difference among themselves ($p > 0.05$). The mean hearing improvement, 3 months following the procedure was almost similar among the three groups ($p = 0.73$). Furthermore, direct FGF application showed no adverse effects as compared to FGF via Gelfoam which presented a risk for granulation tissue and scarring and a risk of myringitis that should not be overlooked. In conclusion, FGF applied directly to large tympanic membrane perforations proved to be a safe, quick and inexpensive healing technique.

In an attempt to make use of the previously proven healing properties of b-FGF (Acharya et al. 2015), selected a small cohort of pediatric patients to specifically demonstrate the usefulness of that technique on children. In his pilot study, Acharya followed the same technique as the one used by his predecessor Kanemaru but with a small modification. It was noticed that the fibrin glue cap, used by Kanemaru to seal the gelatin sponge, underwent liquefaction prompting the failure of Acharya primary procedures. Thus, the fibrin glue was replaced by one drop of cyanoacrylate. 12 patients (6–16 years) with chronic TMP (persistent for more than 3 months) were treated with a single application of b-FGF solution (at a concentration of 21,000 IU/5 ml) and then a regular follow-up for at least 1 year. In this study, there was no control group and results were compared to a standard myringoplasty. An overall 83 % successful closure of TMP was observed with a significant hearing improvement in 80 % of them (t-test $p = 0.024$). With no significant adverse effects, b-FGF proved again its superior regenerative power in an age group where hearing loss can have severe dramatic outcomes that ought not to be underestimated.

11.3.3.3 Hyaluronic Acid

Hyaluronic acid is a naturally occurring extracellular polysaccharide, its value in tympanic membrane healing stems from its influence on rapid restoration of the tissue components. This by itself leads to less scar tissue formation and subsequently to an enhancement of both the structure and the function of the healed tympanic membrane (Hellstrom and Laurent 1987).

In an in vitro study on several biomolecules (Teha et al. 2013), talked about a "Dual signaling" between the extracellular matrix (ECM) and the growth factors, that takes place during an 'in vivo' wound repair. For investigating this effect, the Hyaluronic acid (HA) along with vitronectin and TGF-alpha as well as IL-24 were used, either alone or in combinations, to depict their effects on multiple relevant parameters related to human tympanic membrane derived keratinocytes. Results revealed that the combination of TGF-/HA is the most efficacious in stimulating both migration and proliferation via an epidermal growth factor ERB1 receptor activation and without modification of the epithelial phenotypes. Migration increased by 27 % ($p < 0.05$) and proliferation by 39 % ($p < 0.05$) compared to controls. In this study, authors emphasized on the complexity of tympanic membrane keratinocyte

wound healing process and the importance of keeping the ECM/Growth factor interaction into consideration when assessing for in vivo therapeutic potentials (Teha et al. 2013).

Ozturk et al. (2006) examined the effect of HA on traumatic tympanic membrane perforation. 24 rats were subjected to a bilateral tympanic membrane perforation using a 20 gauge needle and then randomized into 2 study groups: For the right perforated tympanic membrane, one group received an esterified form of HA (Merogel packing), while the other group received daily topical HA administration, the left perforated ears were used as controls. Both study groups revealed significantly greater closure rates compared to the control, on postoperative day 7 ($p<0.05$) as well as an increased amount of VEGF, FGF, lymphocytes, and collagen fibrils ($p<0.05$). Having obtained similar results between Merogel and daily topical HA treatment ($p>0.05$), authors concluded that a single Merogel administration would be a more practical option for both patients and otolaryngologists.

In another series of studies of TMPs, Sayin et al. (2013) used HA ester patching for 155 subjects. Patients were divided into group A with spontaneous closure group (control), and group B to which HA ester (Epifilm otologic lamina) was added and appropriately sealed the perforation dimension. Both groups received oral antibiotics for 5 days. After several follow up examinations up until 6 months post perforation, audiometric evaluations revealed a significant difference between the two groups for perforation closure time; Group B with a closure time of 6.61 ± 4.59, had a shorter closure time than Group A with a closure time of 10.6 ± 5.23 weeks ($p=0.001$). Whereas such a significant difference wasn't observed when comparing closure rate; closure rate for Group A was 85.6 % versus Group B 94.8 % ($p=0.129$). On the other hand, contrary to what has been observed with the aforementioned studies, (Prior et al. 2008) did not reach any eventful results when using the same HA ester (Epifilm) on patients with symptomatic tympanic membrane perforations. Following 6 weeks post-op, 5 patients to which the Epifilm was inserted showed no improvement neither in the perforation size nor in the hearing acuity which led to the suspension of the study. The authors concluded that additional revisions should be considered to understand the reasons for this failure (Prior et al. 2008).

11.3.3.4 Platelets Derived Growth factors (PDGF)

The use of PDGF was assessed in a study of (Yeo et al. 2000) whereby an excision was performed on the posterior aspect of the pars tensa of the TM in the ears of 20 rats. The right TMs of the rats constituted the experimental group and were treated with PBS containing 2 μg of PDGF-AA and 1 % bovine serum albumin, in comparison to the TMs in the left ear which were treated with PBS and 1 % bovine serum albumin and were taken as controls. A complete healing was observed on the 5th day of treatment for all PDGF treated tympanic membranes. However, only 75 % of the control closed by the 15th day with a histological picture revealing a weaker

connective tissue layer as compared to the PDGF treated TMs which had a relatively stronger fibrous layer. Sang and colleague (2000) concluded that not only did PDGF enhance the healing process but it also improved connective tissue growth. Despite the encouraging results, human studies were further needed.

In a later study casting doubt on the effectiveness of this biomolecule, Roosli and coworkers in 2011 found in 10 patients with chronic suppurative otitis media no advantage of topical PDGF in closure of human tympanic membrane perforations, versus a control group of 10 patients subjected to placebo, both treatments were applied weekly for 6 weeks. Complete closure of the perforation was same in both groups and did not differ significantly ($p = 1$), leading the authors to discard the usefulness of PDGF as a promising alternative to surgical treatment of tympanic membrane perforations.

11.3.3.5 Epidermal Growth Factors (EGF) and Pentoxifylline

Many studies, proved that EGF which normally exists in low concentrations in the uninjured TM has a significant contribution in TM wound healing repair by enhancing keratinocyte proliferation and migration (Guneri et al. 2003; Santa Maria et al. 2010) used acute traumatic tympanic membrane perforation models from 30 male albino rats to assess the effect of HA, EGF and Mitomycin C (Mit C) on TM healing. Following a bilateral perforation, the animals were split into groups of 10. A daily application of a treatment; HA (group A), EGF (group B), or Mit C (group C) to the right ears, whereas the left ears were used as control. When comparing histologic parameters in the EGF treated ears to the contralateral ear's histologic parameters no significant differences were noted, however, the mean closure time was significantly shorter with the EGF treated ears as compared to the closure time of the contralateral ears (7.4 ± 1.6 days for EGF treated ears versus 15 ± 1.6 days for the control, $p = 0.0432$) (Guneri et al. 2003).

About a decade ago, the combination of Pentoxifylline with EGF was assessed (Ramalho and Bento 2006). Chinchilla models were subjected to subacute tympanic membrane perforations and divided into 4 study groups: the first treated with topical EGF, the second treated with oral pentoxifylline at 10 mg/kg every 12 h for 10 days, the third combining both treatments together, topical EGF plus oral pentoxifylline, and a control group treated with topical distilled water. Results showed that the addition of pentoxifylline proved to be of no benefit to the healing process observed with the topical EGF treatment. In fact such an addition resulted in 1.8 times lower healing rate when compared to the EGF study group healing rate: Topical EGF group healing rate was 30.3 % whereas topical EGF+ Pentoxifylline group healing rate was 16.5 %.

The authors concluded that the addition of pentoxifylline to the EGF treatment not only did not prove to have a synergistic effect but suggested to have negatively interfered in the healing process of subacute tympanic membranes perforations (Ramalho and Bento 2006).

11.4 Stem Cells

The therapeutic use of stem cells is gaining a great momentum; it is believed to be the gold mine of science. These cells are implicated in the healing of wounds in general due to their proved regenerative capacities (Ghieh et al. 2015). Von Unge et al. in 2002 tested the healing capacity of stem cells in fresh tympanic membranes in Mongolian gerbils. Functional and morphological measures of the TMs were assessed. In this study, 5 adult Mongolian gerbils were used in a Moire interferometry group and 9 were used in a fluorescence microscopy group. Under general anesthesia and otomicroscopy, the inferior portion of the pars tensa was perforated on the left and right tympanic membranes. The perforation of the left tympanic membrane was treated with a droplet containing $<1 \times 10^6$ cells diluted in 5 μl of a physiological NaCl solution. Two types of stem cells were used; embryonic mouse cell line WW6 for the first group and E.P.842BF6 cell line for the second group. The cells were tagged with green fluorescent protein (GFP) and stored at −70 °C. The right ears were reserved as controls (Von Unge et al. 2003).

In the Moire interferometry group the mechanical stiffness of the tympanic membrane was assessed by measurements of the deformation during pressurization using a video interferometer. Sequences of static pressure in steps of 20 daPa up to 200 daPa were used. In the fluorescence microscopy group the temporal bones were dissected so that the entire surface of the tympanic membrane pars tensa could be visualized with a fluorescence microscope.

All five stem cell treated TMs closed in 3 days, whereas only 2 out of 5 closed in the control. Three of the five stem cell treated tympanic membranes ruptured at the pressures 80, 120, and 160 daPa respectively, whereas the other two didn't rupture at all. In the control group, only one tympanic membrane did not rupture.

The displacement patterns under pressurization for both groups where similar, and the peak displacements calculated were slightly less in the stem cell group than in the control. In the fluorescence microscopy group, GFP labeling was observed in the region of the perforation of 3 out of 9 ears of the stem cells treated TMs, whereas GFP labeling was less distinct in the control group (Von Unge et al. 2003). In conclusion, there were better and higher healing scores in the stem cell treated groups. The authors concluded by claiming very promising results (Von Unge et al. 2003).

Another study was conducted by A. Rahman et al, in 2007. The study assessed the acute and long term effects of stem cell treatment on acute TMPs. In this study, 20 Sprague-Dawley rats were divided into 2 groups, A and B. Tau-GFP labeled mouse embryonic stem cells were dispensed at a rate of 10,000 cells/μL in each application (Rahman et al. 2007). Then myringotomy was performed bilaterally under general anesthesia using a KTP laser beam under otomicroscopy. The incision was performed in the postero-superior quadrant of the pars tensa. The stem cells were applied to the right side whereas the left side was left as a control. The animals in group B were treated in the same way. In this group an immunosuppressant (Cyclosporine) was given every other day until 2 weeks. TMs of both groups were monitored for the presence of a perforation, blood clot, infection,

myringosclerosis and thickened TM (Rahman et al. 2007). In the control ears, the closure time was less than the stem cell treated side, regardless of Cyclosporine treatment or not (Rahman et al. 2007). The TMs of both groups showed no blood clots, infection or thickening. At 1 month, a scar was obvious in both groups, however, at the end of the study, all TMs were scarless. Under light microscopy, similar thickness was detected in both groups (Rahman et al. 2007).

In another study also conducted by A Rahman et al. in 2008, using mesenchymal stem cells (MSC) in acute and chronic TMPs, the mechanical stiffness of the healed TMs was measured; both short and long term (Rahman et al. 2008). The study used Sprague-Dawley rats where laser perforations of different diameters were made to the right TMs with KTP laser beams under otomicroscopy. The left TMs were left as controls. Short-term and long-term studies were conducted. In the short-term study, TMs were examined for the healing pattern three times weekly for 2 weeks while in the long-term study TMs were examined twice weekly for 6 months. MSCs treatment was also used on a chronic TMP model. After bilateral myringotomy, a solution of hydrocortisone was instilled around the perforation for 10 consecutive days to make it chronic.

Concerning the acute perforations, results showed that all had similar appearances to controls and closed between 9 and 14 days post myringotomy (Rahman et al. 2008). The pars tensa appeared homogenous, the thickness was almost constant and, the 3 layers of the TM were well identified using transmission electron microscopy. In the long-term study, myringosclerosis was not detected in any of the TMs (Rahman et al. 2008).

In the chronic group, 4 out of 10 ears treated with MSCs had closed perforations, compared to 1 out of 10 in the control ears (Rahman et al. 2008). However, a morphological examination on dissection microscopy showed that the cavities of the ears treated with MSCs were all filled with amorphous material. The major finding in the study is the 40 % closure rate in the MSCs treated TMs compared to 10 % in control ears as determined through otomicroscopy (Rahman et al. 2008).

Another study by Knutsson et al. in 2011 aimed to localize the progenitor stem cells in the human tympanic membrane. As we know, the outer epithelium of the tympanic membrane initiates the closure of a perforation and may act as a scaffold for mesenchymal cell ingrowth and collagen fiber restoration. There are currently no stem cell markers that unequivocally identify interfollicular epidermal stem cells (Alonso and Fuchs 2003; Terunuma et al. 2007). The best marker to date, with the highest sensitivity and specificity, is α6-integrin (Webb and Kaur 2006). Other antigens that have been used are β1-integrin and cytokeratins 15 and 19 (Kaur 2006; Webb and Kaur 2006).

In this study, 5 normal human tympanic membranes with normal preoperative appearance were removed from patients undergoing translabyrinthine surgery (Knutsson et al. 2011). After being stained and washed, all the specimens, except 1 on each slide (negative control), were incubated with 1 or 2 of the primary antibodies (Knutsson et al. 2011). The negative controls were incubated with 2 % bovine serum albumin (BSA). Pooled antiserum from rabbits hyper immunized with specific human α6-integrin was used as the primary antibody against α6-integrin.

Mouse monoclonal antibodies specific for human β1-integrin and CK19 were used as the primary antibodies against β1- integrin and CK 19, respectively. Counterstaining the keratinized epithelial layer was achieved through a cytokeratin antibody to detect the presence of cytokeratins 4, 5, 6, 8, 10, 13 or 18. After repeated washing with PBS the specimens were incubated with the secondary antibodies for 2 h at room temperature in the dark (Knutsson et al. 2011). Then five regions were investigated: the umbo region, the region along the handle of the malleus, the intermediate portion of the pars tensa, the annular region and the skin of the medial portion of the external ear canal. In addition, the umbo region was investigated using laser confocal microscopy. Bright-field and fluorescence images were obtained (Knutsson et al. 2011).

Results showed that the thickness varied from 5 to 10 μm in the middle portion of the pars tensa and the specimens were negative for β1-integrin, CK19 and α6-integrin. As for the umbo, the epithelial thickness was approximately 20 μm. Most of the epithelium was positive for the β1- integrin and CK19, whereas the staining for α6-integrin was positive only in the basal portion of the keratinized epithelium. They were elongated, with an orientation that was slightly inclined from perpendicular to the basal lamina.

In the handle of the malleus, the epithelium had a thickness of 25 μm. All its layers were positive by immunostaining for β1-integrin and CK19, whereas α6-integrin was only in the basal layer (Knutsson et al. 2011). In the annular region, the epithelium stained positive for β1-integrin and CK19. Again, the staining for α6-integrin was positive only in the basal layer. As for the skin, the thickness of the epidermis was approximately 15 μm and the staining pattern was the same (Knutsson et al. 2011). In conclusion, possible progenitor cells could be present in the umbo, the annular region and along the handle of the malleus (Knutsson et al. 2011).

11.5 Conclusion

Throughout history and until now, the gold standard treatment for tympanic membrane perforations was conventional surgery or self-healing, but with the development of tissue engineering and the tendency towards minimal invasive techniques, several alternative methods were tested in the regeneration of tympanic membrane perforations. The use of biomolecules like PDGF, Hyaluronic acid and several others have shown promising results. Moreover, scaffolding materials like silk, Chitosan and calcium alginate have given excellent results with less side effects in the treatment of TMPs. The main focus of this review article was to emphasize on the documented role of stem cells for the past decade in the regeneration of TMPs. Stem cells have been widely used recently in tissue healing. In the 4 articles reviewed in our study stem cells were used in different forms with or without combination with other factors to study their effect on perforations. Some results were very promising especially in chronic TMPs, in the study done by A. Rahman et al. in 2008. This study showed a remarkable rate

of healing in induced chronic TMPs in rats (around 40 %). Despite the encouraging results, many questions still remain unanswered including the availability of the appropriate animal model. Will we be able eventually to replace surgical intervention completely with office-based stem cell therapy? Will stem cells be available as simple ear drops that the patient can buy? Stem cell therapy in TMPs is still a very young and fresh alternative that needs further research in order to achieve its goal in an optimal way.

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Chapter 12
Stem Cell Therapy for Retinal Disease Treatment: An Update

Vamsi K. Gullapalli and Marco A. Zarbin

12.1 Introduction

Retinal degenerative conditions lead to loss of visual function due the inability of a mammalian retina to repair or regenerate itself to a fully functional state. Use of stem cells to restore the anatomy and function of a degenerating retina, and thus vision, is an appealing concept. The most common of these conditions include age-related macular degeneration (AMD), retinitis pigmentosa (RP), and Stargardt disease (SD) (Zarbin 2016).

AMD affects 1.75 million persons in the USA and is the leading cause of blindness in people over the age of 55 years in the USA and Europe (Wong et al. 2014). Central vision is affected in AMD due to progressive degeneration of retinal pigment epithelium (RPE), the underlying choriocapillaris and the overlying photoreceptors (PRs) leading to atrophic patches of outer retina (GA, geographic atrophy) (Zarbin 2016). Central vision also can be affected by growth of abnormal blood vessels (CNV, choroidal neovascularization) under the RPE and retina. There is no proven therapy for GA, but there is effective drug therapy for CNV (Heier et al. 2012; Rosenfeld et al. 2006).

RP and SD are inherited retinal degenerations that cause vision loss in childhood or young adulthood (Parmeggiani 2011). In RP, several different mutations affecting the RPE or photoreceptors (PRs) lead to progressive degeneration of the outer retina throughout the eye causing loss of peripheral and central vision. RP affects 100,000 persons in the USA. SD has a prevalence of 1:10,000 births and is the most common inherited juvenile macular degeneration. Most cases are autosomal recessively

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transmitted and involve a mutation in PRs that causes progressive loss of central vision (Genead et al. 2009). There are no proven treatments for either RP or SD that might slow down the cell loss or restore lost vision.

While gene therapies and drug therapies are being explored as potential treatments for these conditions (Ashtari et al. 2015; Jacobson et al. 2015), none of them would be capable of restoring the PRs and RPE that are lost. Thus, in these cases, replacing the lost cells is an attractive concept that has been explored in animal and human studies. This article provides a brief overview of the use of stem cells in retinal degenerations.

12.2 Goals of Stem Cell Therapy: Rescue and/or Replacement

The goal of stem cell therapy is to either to "rescue" the surviving retinal cells (by providing the necessary support or generating neurotrophic agents) and/or to "replace" the cells that have degenerated. While the concept underlying replacement is straightforward, it became evident from early studies in animals that transplanting retinal cells has a positive effect on the survival of the adjacent cells as well as cells *at a distance* from the site of the transplant. For example, in Royal College of Surgeons (RCS) rats, a model for some forms of human RP, a mutation in transmembrane proto-oncogene tyrosine-protein kinase MER (MertK) in RPE causes poor phagocytosis of shed PR outer segments that subsequently causes degeneration of PRs (D'Cruz et al. 2000). Transplanting normal RPE had a positive effect not only in the immediate vicinity of the transplant site (by *replacing* the ineffective RPE) but also preserves PRs as far as away from the transplanted RPE as 1400 μm (Lund et al. 2001; Vollrath et al. 2001). This benefit was not due to migration of the transplanted cells and points to a trophic effect of the transplant. Indeed, RPE cells are known to produce several PR trophic factors (Kolomeyer and Zarbin 2014; Sun et al. 2015). The distinction may not simply be semantics. If only the outer segment (OS) of a PR has degenerated, for example, and rescue allows the OS to regenerate, then the goal of visual restoration is achieved in a less complicated way without the struggle of reconnecting a transplanted PR with the host retina (Sakai et al. 2003; Zarbin 2016).

12.3 RPE and PRs from Stem Cells

RPE can be harvested from human donor eyes, but they neither grow robustly, nor do they survive well in humans after transplantation. RPE derived from fetuses fare better, but ethical concerns as well as the limited ability to generate large numbers of genetically normal donor cells with serial passage prevent widespread evaluation and use. Stem cells, by nature of their virtually unlimited self-renewal and pluripotency, are a more attractive source for donor tissue.

Table 12.1 Sources of stem cells for retinal disease treatment

Cell type	Example of therapeutic cell type	Advantages	Disadvantages
Embryonic stem cell (ESC)	ESC-derived retinal pigment epithelium (RPE)	Pluripotency Grown relatively easily	Likely to be rejected if donor is allogneic
Adult stem cell	Bone-marrow derived stem cells Neural precursor cells	Multipotency Not rejected if transplanted into donor	Can be relatively hard to Harbors disease-causing genes of donor harvest
Induced pluripotent stem cell (iPSC)	iPSC derived RPE	Pluripotent Grown relatively easily Probably not rejected when injected into the donor	May retain epigenetic features of cell type of origin Harbors disease-causing genes of donor

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Embryonic stem cells (ESCs), derived from the inner cell mass of the blastocyst, can differentiate into cells of ectoderm, mesoderm, and endoderm (Reubinoff et al. 2000; Thomson et al. 1998). Further downstream in the differentiation of the retina is an intermediate cell type with relative reduced proliferative capacity, the multipotent retinal progenitor cell (RPC) (Luo et al. 2014; Marquardt 2003) that could also be a potential source of RPE and PRs. These cells have also been isolated from fetal and adult human eyes (Carter et al. 2007; Coles et al. 2004; Mayer et al. 2005; Yang et al. 2002; Blenkinsop et al. 2013).

Stem cells can be derived from adult tissues; multipotent stem cells have been found in various organs (Gage 2000; Weissman 2000), including the eye (Saini et al. 2016; Salero et al. 2012). In addition, stem cells isolated from a particular tissue can be induced to differentiate into an unrelated tissue. For example, neural stem cells can be induced to develop into muscle.

Pluripotent stem cells can also be generated by somatic nuclear transfer from an adult/fetal/neonatal cell into an unfertilized oocyte (Chung et al. 2014; Tachibana et al. 2013; Yamada et al. 2014), or by transfection of a differentiated adult cell with transcription factors that reactivate developmentally regulated genes, so called induced pluripotent stem cells (iPSCs) (Park et al. 2008; Takahashi et al. 2007) (see Table 12.1). Genetically matched cell lines might thus be generated for autologous transplants (Yabut and Bernstein 2011).

Protocols have been developed to derive retinal cells from ESCs (Osakada et al. 2008; Lamba et al. 2006; Yanai et al. 2016). These cells can rescue PRs in RCS rats (Schraermeyer et al. 2001) or migrate into rabbit retina and express PR markers such as S-opsin and rhodopsin (Amirpour et al. 2012). RPE cells also have been generated from ESCs (Gong et al. 2008; Idelson et al. 2009; Klimanskaya et al. 2004; Lund et al. 2006). These cells also rescue PRs in RCS rats (Lund et al. 2006) and express RPE characteristics including ion transport, resting membrane potential, transepithelial resistance, and visual pigment recycling (Bharti et al. 2011; Maeda et al. 2013).

Other potential sources have been explored. In situ RPE have been recently found to contain a small population of multipotent cells (RPE-ESC) that can be cultured (Saini et al. 2016; Salero et al. 2012) although they may not have the same expansion capability as ESC- or iPSC-derived RPE. Bone marrow-derived stem cells (mesenchymal stem cells) have also been used to generate RPE (Arnhold et al. 2006; Mathivanan et al. 2015). By using surface markers to select the stem cells that have the potential to differentiate into RPE and then co-culturing with mitomycin C-inactivated RPE cells, Mathivanan and coworkers showed that these cells exhibit some of RPE markers and are capable of rescuing PRs after transplantation into RCS rats (Mathivanan et al. 2015). The above two sources may have limitations due to the number of cells that can be derived from them and the lack of complete characterization of these cells.

Can stem cells be differentiated into fully functional RPE and PRs? As noted above, RPE cells that have been derived from ESCs and iPSCs need to exhibit proper ion transport, membrane potential, ability to phagocytose shed PR OSs, polarized vascular growth endothelial growth factor secretion (to maintain normal subjacent choriocapillaris anatomy), visual pigment recycling, and gene expression profiles similar to those of in situ healthy RPE. Expression of these features has varied from lab to lab. A thorough and comprehensive group of functional tests to ascertain the extent of stem cell-derived RPE differentiation has been proposed (Bharti et al. 2011). Using current manufacturing techniques, stem cell-derived RPE can perform the expected functions after transplantation into rodent models of retinal degeneration (Kamao et al. 2014; Maeda et al. 2013; Li et al. 2012; Tsai et al. 2015).

12.4 Experimental Studies and Challenges

Table 12.2 lists ongoing human stem cell trials for retinal degenerative diseases. Stem cells being used include iPSC-RPE, ESC-RPE, iPSC-neural precursor cells (NPCs), bone marrow-derived stem cells, and human central nervous system derived stem cells among others. It is too early to judge the outcome of these sources of tissue. A number of challenges remain that may hinder a successful outcome. Growth arrest due to rapid telomere shortening, chromosomal DNA damage, and increased cyclin-dependent kinase inhibitor 1 (p21) expression (Feng et al. 2010; Kokkinaki et al. 2011), for example, can limit the success of iPSC transplant survival and function.

12.4.1 Stem Cells for Human Transplantation

Generating adequate stem cells in an efficient, rapid, and safe manner would permit widespread use. Phenotypic instability or altered gene expression during serial passaging in culture, including up-regulation of oncogenes, might occur and mandate careful monitoring of the manufacturing process (Klimanskaya et al. 2004; Anguera et al. 2012; Shen et al. 2008).

Table 12.2 Current human cell therapy trials for retinal diseases

Disease (Clinicaltrials.gov Identifier)	Phase	Cell type transplanted	Center (PI)	Sponsor
AMD (NCT00874783)	Observational	iPSCs	Hadassah Medical Organization (Reubinoff)	Hadassah Medical Organization
AMD-GA (NCT02286089)	I/II	ESC-RPE	Hadassah Ein Kerem University Hospital (Hemo)	Cell Cure Neurosciences
AMD-GA (NCT02016508)	I/II	Bone marrow-derived SCs	Al-Azhar University (Safwat)	Al-Azhar University
AMD-GA (NCT02590692)	I/IIa	ESC-RPE on a polymeric substrate (CPCB-RPE1)	Retina Vitreous Associates Medical Group (Rahhal) USC Keck School of Medicine (Kashani)	Regenerative Patch Technologies
AMD-CNV (NCT01691261)	I	ESC-RPE on a polyester membrane	University College London (Pfizer)	Pfizer
AMD-GA or CNV (NCT02464956)	Observational	Autologous iPSC-RPE	Moorfields Eye Hospital	Moorfields Eye Hospital NHS Foundation Trust
AMD (NNCT01920867)	Interventional	Bone marrow-derived SCs	Retina Associates of South Florida (Weiss)	Retina Associates of South Florida
AMD-GA (NCT01736059)	I	Bone marrow-derived CD34+ SCs	University of California, Davis (Park)	University of California, Davis
AMD-CNV	Interventional	Autologous iPSC-RPE	Riken Institute for Developmental Biology (Takahashi)	Riken Institute for Developmental Biology

(continued)

Table 12.2 (continued)

Disease (Clinicaltrials.gov Identifier)	Phase	Cell type transplanted	Center (PI)	Sponsor
Stargardt disease (NCT01345006, NCT02445612, NCT01469832)	I/II	ESC-RPE (MA09-hRPE)	Jules Stein-UCLA (Schwartz) Wills Eye Hospital (Regillo) Bascom Palmer Eye Institute (Lam) Moorfields Eye Hospital (Bainbridge)	Ocata Therapeutics
AMD-GA (NCT01344993, NCT02563782, NCT02463344)	I/II	ESC-RPE (MA09-hRPE)	Jules Stein-UCLA (Schwartz) Wills Eye Hospital (Regillo) Mass. Eye and Ear Infirmary (Eliott) Bascom Palmer Eye Institute (Rosenfeld)	Ocata Therapeutics
AMD-GA (NCT01632527)	I/II	HuCNS-SC	Retina Foundation of the Southwest (Birch)	StemCells
AMD-GA (NCT01674829)	I/II	ESC-RPE (MA09-hRPE)	CHA Bundang Medical Center (Song)	CHA Bio and Diostech
Stargardt disease (NCT01625559)	I	ESC-RPE (MA09-hRPE)	CHA Bundang Medical Center (Song)	CHA Bio and Diostech
Myopic macular degeneration (NCT02122159)	I/II	ESC-RPE (MA09-hRPE)	Jules Stein-UCLA (Schwartz)	Ocata Therapeutics
AMD-GA (NCT01226628)	I	CNTO 2476 (umbilical tissue-derived cells)	Wills Eye Hospital (Ho)	Janssen Research and Development

Table 12.2 (continued)

Disease (Clinicaltrials.gov Identifier)	Phase	Cell type transplanted	Center (PI)	Sponsor
AMD-GA or CNV (NCT01518127)	I/II	Autologous bone marrow-derived SCs	University of Sao Paulo, Brazil (Siqueira)	University of Sao Paulo
RP and cone-rod dystrophy (NCT01068561)	I/II	Autologous bone marrow-derived SCs	University of Sao Paulo, Brazil (Siqueira)	University of Sao Paulo

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12.4.2 Cell Delivery

Various techniques have been used and are being explored to allow for an efficient and effective delivery of transplanted cells to the retina. The transplant cells may be injected into the vitreous (Park et al. 2015) or into the subretinal space (Schwartz et al. 2015; Li et al. 2012) as a cell suspension (intravitreous or subretinal delivery) (Diniz et al. 2013) or as a sheet of cells (subretinal delivery) (Kamao et al. 2014) with or without a biocompatible scaffold (Hsiung et al. 2015; Redenti et al. 2008; Tao et al. 2007; Tucker et al. 2010; Stanzel et al. 2014). Advantages of a cell suspension include ease of preparation and ease of delivery through a small retinotomy. However, there is little control of how transplanted cells reorganize in the subretinal space. The cells may form multilayers; they may not be polarized in the correct way; and the cells, especially RPE transplants, will need to re-attach to an abnormal Bruch's membrane surface. Cells sheets, on the other hand, allow for placement of properly polarized cells (e.g., apical villi of RPE facing PR OSs) that can start functioning immediately, and the scaffold that holds the cell sheets may allow for integration of growth factors or immunomodulatory factors to promote transplant survival and function. The scaffold may also confer some degree of protection against Bruch's membrane-induced cell death. Placement of cell sheets, however, requires a larger retinal opening that could potentially lead to egress of transplanted cells or retinal detachment after surgery.

Different potential scaffolds to support RPE sheet transplants are being explored (Kundu et al. 2014; Nazari et al. 2015). These include vitronectin-coated polyester membranes (Carr et al. 2013) and parylene C scaffolds manufactured using nanotechnology (Lu et al. 2012, 2014).

Transplants of PR sheets have consisted of either PR sheets, full thickness retinal sheets, or retina-RPE sheets (Assawachananont et al. 2014; Radtke et al. 2008;

Huang et al. 1998; Radtke et al. 1999). While the full thickness retina can still establish synaptic connections and restore visual responses in rats (Seiler et al. 2010), for example, whether patients would experience useful visual improvement given the altered anatomy of a "double" retina is not clear. How a suspension of PRs compares to a sheet of pure PRs is also not known.

12.4.3 Transplant Survival, Differentiation, and Integration

For transplantation to be successful, RPE must survive in the subretinal space, reattach to the underlying Bruch's membrane (the structure on which RPE normally reside), be polarized so that PR OSs can be phagocytosed by the apical villi, and establish an outer blood-retinal barrier (e.g., via tight junctions between adjacent RPE cells). PR transplants will need to survive, extend axons to form synapses with the host bipolar cells one side, and extend OSs towards the native RPE cells on the other side. Loss of PRs due to mutations or retinal detachment leads to subsequent synaptic rewiring between other interconnected retinal cells (Khodair et al. 2003; Lewis et al. 1998; Jones et al. 2003). In other words, a mere integration of the transplanted PRs with the downstream bipolar cells alone may not be sufficient for complete visual recovery due to synaptic rewiring of the retina that occurs once host PRs have degenerated.

Results from preclinical models of RP indicate that if one transplants suspensions of rod PRs into the subretinal space, the cells need to be of a specific developmental stage for the transplant to have the highest chance of success. Specifically, post-mitotic rod precursors that express the rod-specific transcription factor, Nrl, yet are morphologically immature, seem to give the best results (MacLaren et al. 2006; Pearson 2014; Pearson et al. 2012; Akimoto et al. 2006). In addition, with current techniques, it is important to transplant a large number of cells (~200,000) to achieve improved visual function. Wild-type rod PR precursors generated from fetal tissue and transplanted into rd1 mice (which lack phosphodiesterase-6-beta (Pde6b) and exhibit rapid rod PR death after birth) express Pde6b in an appropriately polarized fashion, exhibit abnormally short OSs, and support improved visual function (Singh et al. 2013). In the rd1 recipients, the host bipolar and Muller cells extend processes into the PR graft and appear to make synaptic contact with the donor PRs (Singh et al. 2013). Gonzalez-Cordero and co-workers harvested developing PRs from optic cups generated from ESCs in vitro and noted that best integration with host rd1 retina occurs when these PR precursors are still immature but committed to becoming PRs, which is quite similar to the results observed when using fetal tissue as the source of PR precursors (Gonzalez-Cordero et al. 2013). Host retinal anatomy can modulate the efficacy of PR transplantation. If the host retina has significant PR damage and abnormal anatomy, the transplanted PRs also tend to exhibit abnormal and limited synapse formation (Barber et al. 2013). Glial scarring limits integration in more advanced stages of retinal degeneration, and attenuating the glial barrier helps promote better integration in some types of retinal degeneration

(Pearson 2014; Barber et al. 2013; Pearson et al. 2010; Hippert et al. 2016). An additional barrier may be the external limiting membrane (ELM), which is formed by the junction of Muller cell apical processes and PR inner segments via adherens junctions. In one study, transient ELM disruption using alpha amino adipic acid improved PR precursor integration by ~100% (West et al. 2008; Pearson et al. 2010). Indeed, in retinal degenerations associated with ELM disruption, there is greater integration of transplanted PRs with host retina (Barber et al. 2013).

In principle, it should be easier to achieve clinically successful outcomes after RPE transplantation (for the purpose of "rescue") than after PR transplantation (for the purpose of "replacement") since RPE integrate with host PRs spontaneously. Thus, the only challenge for a successful RPE transplant, apart from the need to control immune surveillance, involves resurfacing an atrophic patch in the foveal area in AMD patients with GA. Transplanted RPE have been shown to survive and rescue PRs in numerous preclinical studies. However, human studies have not resulted in a comparable degree of success (Binder et al. 2007; Gullapalli et al. 2012). RPE survival has been shown to be poor when transplanted onto Bruch's membrane from aged human cadaver eyes or eyes with advanced AMD with GA (Sugino et al. 2011b; Gullapalli et al. 2005). In addition, human Bruch's membrane has been shown to undergo changes resulting from aging including thickening, advanced glycation end-product formation, lipid and protein deposition, and protein crosslinking (Zarbin 2004). As mentioned above, one way to address this issue would be to use scaffolds on which transplanted RPE could be delivered as a differentiated cell sheet in which the scaffold provides a surface conducive to cell survival and prevents contact of the transplant with subjacent host Bruch's membrane. Use of conditioned medium derived from bovine corneal endothelial cells has been shown to improve transplanted RPE survival on human cadaver eyes with GA (Sugino et al. 2011a) by altering cell behavior on this surface. Identification of molecules responsible for this effect might allow development of an adjunct that would improve transplanted RPE cell survival in AMD eyes, even when cell suspensions are used.

12.4.4 Immune Response

The subretinal space is an immune privileged site, i.e., allografts survive longer in this privileged site compared to a non-privileged site such as the subconjunctival space. Native neonatal RPE behaves as an immune privileged tissue, i.e., RPE resist rejection at heterotopic sites (Wenkel and Streilein 2000). Do stem cell-derived RPE behave as immune privileged tissue? ESCs and their derivatives have been shown to escape a host immune rejection for a long time (Yuan et al. 2007). This phenomenon may be due to low expression of human leukocyte antigen (HLA) class I molecules and no expression of class II molecules in their resting or differentiated state (Drukker et al. 2002). ESCs have also been shown to suppress T-cell proliferation (Li et al. 2004). iPSCs, on the other hand, appear to capable of inducing immune rejection (Sohn et al. 2015), even if autologous (Zhao et al. 2011).

Disruption of the blood-retinal barrier can be a significant factor in stimulating the immune response. For example, disruption of native RPE (e.g., using sodium iodate) results in loss of the immune privilege of the subretinal space. Preservation of the barrier diminishes the immune response (Lu et al. 2010). RPE transplants in rabbits require immune suppression for sustained cell survival, (Stanzel et al. 2014) but this result may be due to the merangiotic nature of rabbit retina (i.e., only a choroidal blood supply for most of the retina) (De Schaepdrijver et al. 1989). In contrast, human retina is holangiotic (i.e., the retinal and choroidal circulation provide blood flow to the retina). Activation of the innate immune system can lead to activation of the adaptive immune system, which mediates immune surveillance. As a result, it is important to use surgical techniques and devices that minimize disruption of the blood retinal barrier and that incite acute inflammation.

Microglial activation in the host retina has been attributed to failure of grafts to survive and integrate (Bull et al. 2008; Singhal et al. 2008). Suppression of microglial activation may improve transplant survival and integration (Xian and Huang 2015).

Postoperative immune suppression will likely be needed for RPE transplants, but elderly patients with AMD may not be able to tolerate extended periods of immune suppression (Tezel et al. 2007). Long-acting intravitreal steroid preparations may be of use. It is not clear that PR transplants will require long-term immune suppression as these cells exhibit very low MHC class II expression. However, if full thickness retina transplants are used or if impure PR preparations are used, then transplantation of microglia will probably activate a host immune response.

12.4.5 Tumor Formation

One of the important risks of stem cell transplants is development of tumors. When ESC-derived neural precursors were injected into the subretinal space of rhodopsin knockout mice, 50 % of the eyes developed tumors (teratomas) within 8 weeks (Arnhold et al. 2004). (These mice have a mutation resembling autosomal dominant RP.) When iPSC and ESC mouse lines were compared, there was high incidence of teratoma formation with both of them (Araki et al. 2013). There have been no reported tumor issues with patients with SD and AMD who have received ESC-derived RPE cells (Song et al. 2015; Schwartz et al. 2015). iPSC cell lines may be more prone to genetic instability due to the risk of insertional mutagenesis from use of viral vectors and use of oncogenic factors such as c-Myc during cell production. Use of non-integrating reprogramming methods in the production of iPSC cell lines might reduce the risk (Kang et al. 2015) by increasing genomic stability. Nonetheless, careful sustained monitoring will be needed.

12.5 Conclusion

The concept of transplanting healthy cells into diseased retina to restore vision is appealing. Significant progress has been made during the last 30 years. Preclinical testing has demonstrated the feasibility of cell-based therapy for the purpose of sight preservation as well as sight restoration. This research also has identified obstacles to success including graft survival and differentiation as well as immune rejection. Strides in stem cell research have allowed for expanding the field significantly. Early phase human trials using stem cell-derived donor tissue have also been promising. Continuing research in various aspects of transplantation- establishing cell lines without danger of tumor formation or immune rejection, refining surgical techniques and instruments, and identifying factors that promote cell survival, differentiation, and integration of the transplanted cells, should allow for rapid and continued progress in the field.

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Chapter 13
Stem Cell Applications in Corneal Regeneration and Wound Repair

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Abbreviations

ABCG-2	ATP-binding cassette transporter subtype G-2
ACAID	Anterior chamber-associated immune deviation
ALDH	Aldehyde dehydrogenase
ALK	Anterior lamellar keratoplasty
APC	Antigen presenting cell
ATMP	Advanced therapy medicinal product
ATP	Adenosine triphosphate
CAOMECS	Cultured autologous oral mucosal epithelial cell sheets
CK3	Cytokeratin 3
CK12	Cytokeratin 12
CLET	Cultivated limbal epithelial transplant

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DALK	Deep anterior lamellar keratoplasty
DPC	Dental pulp cell
DMEK	Descemet's membrane endothelial keratoplasty
DSAEK	Descemet's stripping automated endothelial keratoplasty
DSEK	Descemet's stripping endothelial keratoplasty
ECM	Extracellular matrix
EDC	1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride
EnMT	Endothelial-to-mesenchymal transition
eTAC	Early transient amplifying cell
FBS	Fetal bovine serum
HAEC	Human amniotic epithelial cells
HCEnC	Human corneal endothelial cell
HCEnP	Human corneal endothelial progenitor
hIDPSC	Human immature dental pulp stem cell
HvGD	Host-versus-graft disease
GAG	Glycosaminoglycan
GvHD	Graft-versus-host disease
IL	Interleukin
iPSC	Induced pluripotent stem cell
KLAL	Keratolimbal allograft
LESC	Limbal epithelial stem cell
LSCD	Limbal stem cell deficiency
MHC	I major histocompatibility complex I
MHC	II major histocompatibility complex II
MMP	Matrix metalloproteinase
MSC	Mesenchymal stem cell
PG	Proteoglycan
PK	Penetrating keratoplasty
PMC	Post-mitotic cell
PNIPAM	Poly(N-isopropylacrylamide)
ROCK	Rho-associated kinase
SP	Side population
TAC	Transient amplifying cell
TGF-β1	Transforming growth factor-β1
Th1	Type I helper T cell
Treg	Regulatory T cells
TSG-6	Tumor necrosis factor-inducible gene 6 protein
TSP-1	Thrombspondin-1
VEGF	Vascular endothelial growth factor

13.1 Introduction: The Human Cornea

13.1.1 Development

The cornea is the transparent avascular dome-shaped tissue that lies in front of the iris. When the human eye begins its development at the 17th day of gestation, two blastoderm layers play an important role: the ectoderm and the mesoderm (the outer and the middle layers of the blastoderm respectively). By the end of the third week of gestation the optic vesicle forms and fold inwards to create the optic cups. The retina and the lens subsequently develop from thickening ectoderm cells (Barber 1965). The cornea begins as the initial layer of ectoderm that covers the developing lens. The ectoderm cells double in number and proceed to form the corneal epithelium (Sellheyer and Spitznas 1988). When the lens has completed its formation, it separates from the corneal ectoderm, with the space between becoming the anterior chamber. Mesenchymal cells of neural crest origin invade the developing cornea and form an endothelial layer lining the primary stroma. A second mesenchymal invasion targets the primary stroma and the neural crest cells develop into keratocytes that embed in the collagen fibrils (Duke-Elder 1964; Mann 1964; Barber 1965; Hay and Revel 1969). These keratocytes secrete an extracellular matrix, consisting of proteoglycans and collagen types I, V and VI (Hart 1976; Hay 1980). At the seventh month of gestation, the stroma becomes dehydrated, thinner, and transparent while the keratocytes move into their characteristic quiescent phase (Jester et al. 1994).

13.1.2 Anatomy

The cornea covers 15 % of the surface area of the globe (Ruberti et al. 2011). It has an axial radius of 7.8 mm anteriorly and 6.5 mm posteriorly, resulting in an internal concave surface that is steeper than the external convex surface (Freegard 1997). The mean diameter of the cornea is 11.71 mm (Rüfer et al. 2005) and the thickness varies from approximately 520 μm in the middle to 650 μm in the periphery (Bron et al. 1997). The cornea largely consists of highly organized collagen fibrils that form up to 200 sheets of stromal lamellae. The mean interfibrillary distance is 41.5 nm and the diameter of the collagen fibrils ranges from 22.5 to 35 nm, depending on the type of collagen (Sayers et al. 1982). Each lamella is arranged at a right

angle relative to the fibrils in the adjacent lamellae, but within the lamella the fibrils lie parallel to each other and to the corneal plane (Maurice 1957). The stacking of these layers with a range of orientations reinforces the structural integrity of the cornea. The interweaving of the anterior lamellae and termination in the Bowman's membrane accounts for the increased rigidity of the anterior stroma when compared to the posterior stroma. The anterior stromal architecture also accounts for maintenance of the corneal curvature (Müller et al. 2001). Collagen fibrils change in orientation as they approach the limbus and run circumferentially (Meek and Boote 2004). The differing organization of collagen lamellae along the anterior-posterior axis, together with structural changes in the collagen fibrils at the limbus and the variation in corneal thickness, has an impact on the biomechanical characteristics, making the cornea a reasonably strong and resilient construct (Ruberti et al. 2011).

The stroma is the thickest layer of the cornea and is bound by anterior and posterior limiting membranes with their respective cellular layers: the epithelium and endothelium. Peripherally the stroma is bounded by the corneoscleral limbus, a circular zone that forms the junction of the transparent cornea and the opaque sclera. In health, the cornea is completely avascular because even the smallest capillary would interfere with transmission and refraction of light. It is however, one of the most densely innervated tissues in the human body (Müller et al. 2003). Approximately 71 nerve bundles enter the cornea at the limbus, resulting in approximately 605 nerve terminals/mm^2 in the suprabasal layers of the corneal epithelium (Marfurt et al. 2010). Corneal nerves produce trophic factors, increase tear production and modulate the blinking response (Müller et al. 2003). They are predominantly afferent sensory nocireceptors, which transduce chemical, thermal and mechanical stimuli as pain sensations (Belmonte et al. 2004; Kubilus and Linsenmayer 2010). Corneal transparency and integrity are affected severely by disruption of the nerves (Beuerman and Schimmelpfennig 1980).

13.1.3 Histology

The cornea consists of five morphologically distinct layers consisting of three functionally differing cellular layers (Beuerman and Pedroza 1996). The outer layer of the cornea is the epithelium, which is separated from the central stroma by a basement membrane and Bowman's layer (anterior limiting membrane). The endothelium is the innermost layer and it is separated from the stroma by Descemet's membrane (posterior limiting membrane) (Fig. 13.1).

- Epithelium: 50–90 μm thick stratified squamous non-keratinized cell layer
  - Bowmans's layer: 8–14 μm cell-free collagen layer
- Stroma: 500 μm highly organized collagen structure containing keratocytes
  - Descemet's membrane: 5–10 μm thick layer of collagen type IV
- Endothelium: single layer of hexagonal-shaped cells, 4 μm thick in adults

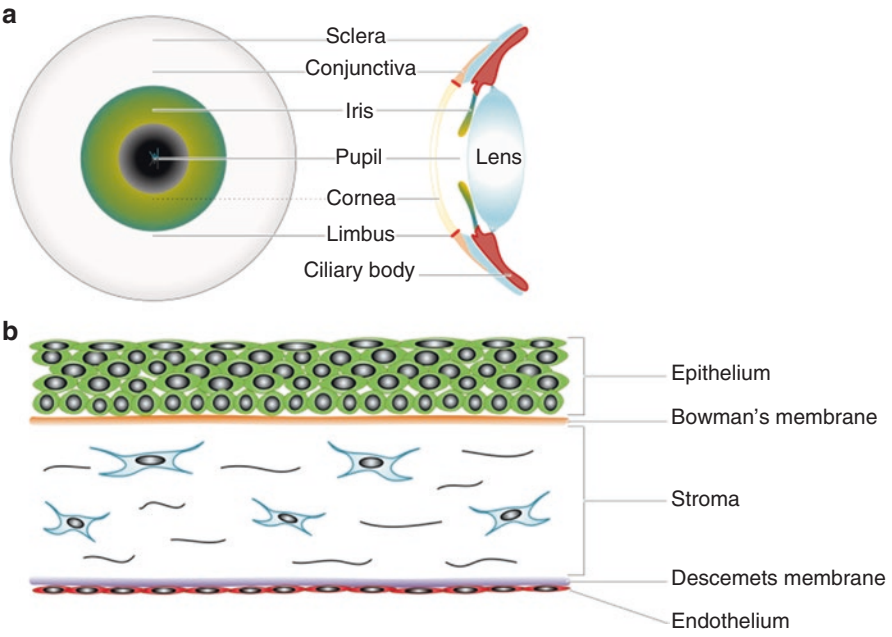

Fig. 13.1 Exterior eye anatomy in frontal and side view (**a**). Anatomy of the cornea with its five distinct layers, the epithelium being the anterior most layer. Keratocytes lie scarcely dispersed in the stromal matrix (**b**)

13.1.3.1 Epithelium

The epithelium is the outermost layer of the cornea. The epithelial cells form five to seven layers which are shed continuously, with a turnover of 7–10 days (Hanna et al. 1961). There are three distinct cell types present in the epithelium. The superficial two layers are flattened surface cells that play an important role in tear film stability. The next two to three layers consist of polyhedral wing cells followed by a basal cell layer of tall columnar cells. The basal "Transient Amplifying Cells" are the only cells capable of mitosis apart from the epithelial stem cells and are the source of superficial and wing cells (DelMonte and Kim 2011) (Fig. 13.2). The barrier function and the transparency of the epithelium are ensured by intercellular junctions in the wing cells and basal cells. The basal cell layer also secretes extracellular matrix that forms the basement membrane, a 0.05 µm thick layer composed of laminin and collagen IV (Beuerman and Pedroza 1996).

The Bowman's layer lies between the epithelium and the stroma and consists of an amorphous zone of collagen type I and II. The membrane is acellular and has no capacity to regenerate after injury. The layer is well-developed in higher mammals but seems to be rudimentary or even absent in lower taxa (Merindano et al. 2002; Hayashi et al. 2002).

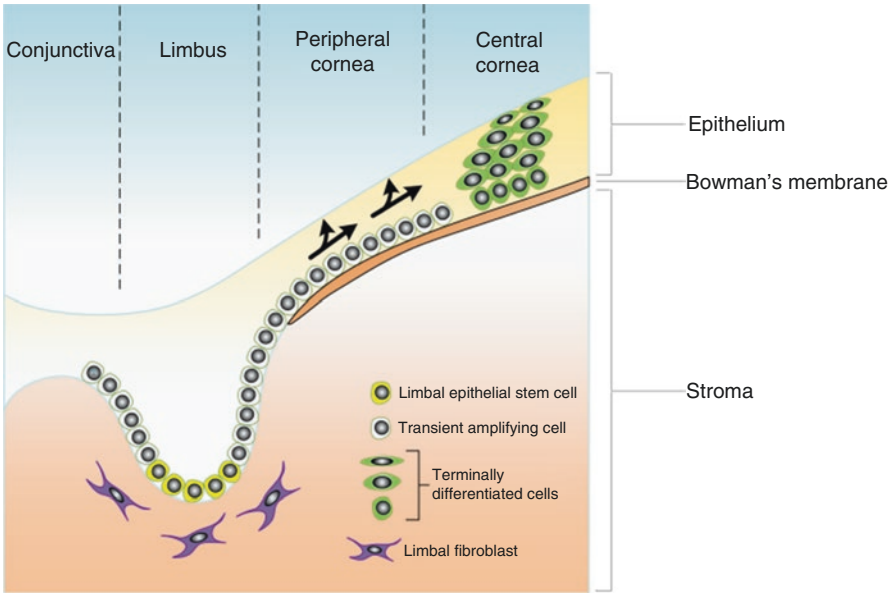

Fig. 13.2 Limbal epithelial stem cells reside in the undulating basal layer of the epithelium. These undulations are also referred to as stem cell niches. Transient amplifying cells divide and migrate towards the central cornea, where they become terminally differentiated epithelial cells, replacing old epithelial cells that are sloughed off into the tear film

13.1.3.2 Stroma

The stroma accounts for approximately 90 % of corneal volume. It is composed of collagen fibrils and glycosaminoglycans (GAGs) with proteoglycan groups (PGs), which are secreted by keratocytes dispersed within the matrix. Keratan sulfate is the most abundant GAG, and accounts for up to 65 % of the total amount of GAGs. The posterior cornea contains more hydrophilic proteoglycans such as keratan sulphate, while the anterior consists mainly of dermatan sulphate which is far less hydrophilic (Müller et al. 2001). Keratocytes are fibroblast-like cells which are mitotically quiescent under normal physiological conditions and occupy 3–5 % of the stromal volume. They produce the extracellular matrix, including GAGs, PGs, matrix metalloproteinases (MMPs) and highly organized collagen structures, that are critical for the transparency of the cornea and the maintenance of the corneal homeostasis (Fini 1999; Du et al. 2005; Hassell and Birk 2010; DelMonte and Kim 2011).

Descemet's membrane beneath the stroma is an acellular layer which serves as a basement membrane for the underlying endothelium. It is continuously synthesized by the endothelium and, unlike Bowman's membrane, has the ability to regenerate after trauma,. The thickness is dependent on age, from 3 μm at birth to a maximum of 10 μm in old age (DelMonte and Kim 2011). The Descemet's membrane is very resistant to invasive events such as infection, chemical damage or enzymatic degradation (Pavelka and Roth 2010). It consists of a different type of collagen than the stroma, namely collagen type VIII (Tamura et al. 1991). In 2013, a new layer was

proposed localized in between the stroma and Descemet's membrane and named the Dua's layer. Dua's layer is a thin air-impervious layer of collagen type I (around 15 μm) that is very biomechanically strong for its thickness (Dua et al. 2013). Since this discovery was only made recently based on clinical findings during lamellar air dissection of the cornea, the presence of this layer has not been widely accepted and its status remains disputed.

13.1.3.3 Endothelium

The endothelium is a single layer of non-proliferating hexagonal cells with a honeycomb-like appearance. The number of cells decreases from 6000 cells/mm^2 in newborns (mean cell diameter 20 μm and thickness 10 μm) to an average of 3000 cells/mm^2 in adults (mean cell diameter 40 μm and thickness 4 μm) (DelMonte and Kim 2011). The cells are attached to each other and to Descemet's membrane by tight junctional complexes and hemidesmosomes. The endothelium and to a lesser extent, the epithelium, play an important role in the maintenance of the corneal deturgescence.

13.1.4 Physiology

Optically, the cornea acts as a lens with a fixed focus. It transmits incoming light to the ocular crystalline lens, which focuses it onto the retina. The cornea provides a refractive power of 43 diopters, which is ¾ of the total refractive power of the eye. The mean refractive index of the cornea is 1.376, which is similar to that of the vitreous (1.336) (Hecht 1987). The physical basis of corneal transparency is still debated (Freegard 1997). Two theories are frequently supported: (i) the lattice theory postulated by Maurice in 1957 and (ii) the theory by Goldman and Benedek that light cannot resolve structures smaller than the dimensions of its wavelength, summarized by Benedek in 1971 (Maurice 1957; Benedek 1971). In order to maintain these optical properties, the cornea must tightly regulate its water content at 78 % (Thiagarajah and Verkman 2002). The relative deturgescent state is maintained by the endothelium and epithelium (Geroski et al. 1985). The cell membranes of the endothelium contain a high number of Na+/K+-ATPase pumps that create an osmotic gradient in collaboration with the intracellular carbonic anhydrase pathway (Stiemke et al. 1991). Fluid from the stroma follows the gradient towards the aqueous humor. The cornea requires energy to maintain the gradient which is generated through the breakdown of ATP from glucose in the active metabolizing layers, the epi- and endothelium. Other nutrients are also needed, such as oxygen to maintain a minimum oxygen tension, which prevents hydration and swelling. The cornea obtains oxygen through the tear film, limbal capillaries and, to a lesser extent, from the aqueous humor. Amino acids also play a crucial role in the turnover of the epithelial cell layers, and are derived from the aqueous humor by passive diffusion.

Immunologically, the eye is a privileged organ and its functions are preserved through self-regulation of inflammation, making corneal transplants the least-rejected of all organ transplants (Streilein 2003; Niederkorn 2006). In the 1940's the concept of immune privilege was proposed by Medawar, but it was only recently that the idea of immunological ignorance was refined and active immune suppression mechanisms were explored (Streilein 2003). Currently there are three mechanisms of immune privilege in the eye which represent different lines of thought: (1) different kinds of barriers exist in the eye: molecular, cellular and anatomical; (2) anterior chamber-associated immune deviation (ACAID) as immunological tolerance; (3) the intraocular microenvironment is immune suppressive (Hori 2008). The cornea is not vascularized nor does it contain lymphoid cells or other defence mechanisms, with the exception of dendritic cells reducing its capacity to prime an immune response (Hamrah et al. 2003). Langerhans cells and lymphocytes are mainly found in the limbus, which is highly vascularized in contrast to the cornea (Vantrappen et al. 1985; Hendricks et al. 1992). Corneal cells (epithelial cells, keratocytes, endothelial cells) lack the expression of major histocompatibility complex II (MHC II) and only express low levels of major histocompatibility complex I (MHC I) (Wang et al. 1987), therefore, it takes considerable time before antigen recognition and effector cell activation take place. Only the peripheral cornea or limbus contains MHC II-positive dendritic cells and macrophages, which cannot be found in other regions of the cornea (Streilein et al. 1979). After corneal transplantation, antigen presentation is carried out by the host antigen presenting cells (APCs) and not by donor-derived APCs (Hamrah et al. 2003). Recognition of donor antigens occur indirectly through CD4 T-cells and the main mechanism of rejection is assumed to be delayed-type hypersensitivity in which minor H antigens are targeted instead of the donor's MHC molecules (Sonoda and Streilein 1992, 1993).

13.2 Stem Cells in the Cornea

13.2.1 Limbal Epithelial Stem Cells

The self-renewing capacity of the corneal epithelium is maintained by slow-cycling corneal epithelial stem cells referred to as limbal epithelial stem cells (LESC) (Davanger and Evensen 1971; Daniels et al. 2001; Secker and Daniels 2009; Pinnamaneni and Funderburgh 2012). LESCs reside in the limbus, more specifically in the palisades of Vogt which form the limbal stem cell niche (Schermer et al. 1986; Cotsarelis et al. 1989). The limbus is the region of the cornea with the highest levels of messenger RNA (Priya et al. 2013). The superior and inferior limbal regions are more densely packed with palisades, which can be seen clinically as radial fibrovascular creases (Goldberg and Bron 1982). The stem cells in the superior and inferior niches are protected by partial covering of the eyelids and melanocytes that reduce exposure to ultraviolet light (Shimmura and Tsubota 1997). Adenosine triphosphate (ATP) binding cassette transporter subtype G-2 (ABCG2) protects the stem cells

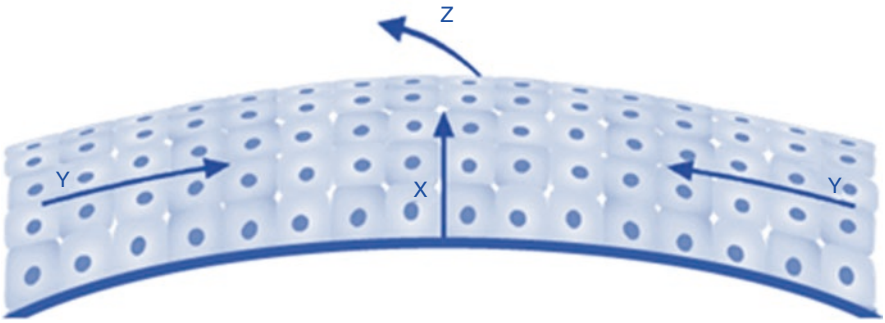

Fig. 13.3 XYZ-hypothesis according to Thoft and Friend (1983). *X* cell proliferation from the basal layer of the epithelium, *Y* centripetal migration during differentiation, *Z* sloughing off of cells at the corneal surface

against phototoxic and oxidative stress-inducing conditions (Kubota et al. 2010). Stromal cells also assist in maintaining the stemness features of the LESC, as recently shown by Mariappan et al. (2010).

LESC remain undifferentiated in a resting state (Secker and Daniels 2009) until epithelial shedding activates the dormant cells (Thoft and Friend 1983; Cotsarelis et al. 1989). The LECSs go through asymmetrical division, each producing one stem cell which remains in the niche, maintaining the cell population, while the other becomes an early transient amplifying cell (eTAC) (Morrison and Kimble 2006; Knoblich 2008; He et al. 2009). The eTACs migrate away from the stem cell niche in a centripetal direction and give rise to late TACs located at the basal layer, on to suprabasal post-mitotic cells (PMCs) and finally terminally differentiated cells located at the surface (Li et al. 2007; Secker and Daniels 2009). This process of migration was postulated in 1983 by Thoft and Friend as the XYZ-hypothesis, **X** being the proliferation at the basal level, **Y** contribution to the cell mass by centripetal migration during differentiation and **Z** designates the loss of cells at the surface (Fig. 13.3).

13.2.2 Mesenchymal Stem Cells in the Stroma

Fibroblast-like cells called keratocytes, lie sparsely scattered throughout the corneal stroma, making contact with one another through extended cytoplasmic processes. Corneal keratocytes are derived from mesenchymal stem cells (of osteogenic origin) which were first described by Alexander Friedenstein in 1968. More recently, multipotent stem cells in the human corneal stroma were described by Du et al. Mesenchymal stem cells (MSCs) lie within the stroma, close to the limbus and express specific markers that are absent in differentiated keratocytes. MSCs can be induced to form the keratocyte phenotype in the presence of certain growth factors such as fibroblast growth factor-2 (Du et al. 2005). They have been shown to be

successful in regenerating the extracellular matrix and in repairing defects in the collagen fibrils of opaque corneas in a mouse model (Du et al. 2009). The ability of MSCs to restore transparency was subsequently confirmed using umbilical cord-derived MSCs (Liu et al. 2010). Since then, many reports on the presence of MSCs in human corneal stroma have followed (Polisetty et al. 2008; Pinnamaneni and Funderburgh 2012; Li et al. 2012), although there is a wide range of approaches described for their isolation, expansion and characterization criteria. In 2012, Branch and colleagues characterized cells isolated from the peripheral cornea and limbus using the minimal criteria for human mesenchymal stem cells as proposed by the ISCT (International Society for Cellular Therapy) in 2006, see Sect. 13.2.4 (Dominici et al. 2006; Branch et al. 2012). Corneal mesenchymal stem cells are immune privileged and have immunosuppressive properties, although far weaker than the capacity of bone marrow-derived MSCs (Patel et al. 2008; Du et al. 2009).

13.2.3 Endothelial Stem Cells

In 2005, Whikehart and coworkers posited the existence of corneal endothelial stem cells (Whikehart et al. 2005). Telomerase activity, a specific characteristic of transient amplifying cells (Ulaner and Giudice 1997), was demonstrated in peripheral regions of the endothelium. Further studies made use of sphere-forming assays to establish proof of the presence of endothelial precursors (Yokoo et al. 2005). Tissue-committed precursors with the capacity for self-renewal were identified in peripheral human corneal endothelial cells and the rate of primary sphere formation was four times greater than that of the central cornea (Yamagami et al. 2007). Following this observation it was proposed that a stem cell niche may be present at the junction of the endothelial cell layer and the trabecular meshwork (Joyce 2003; Whikehart et al. 2005). Cells that may represent transient amplifying cells have been seen, in a human ex vivo model, to migrate from the peripheral junction zone, to form the endothelial cell layer (He et al. 2012). Wounded areas could be supplied by this cell source but migration does not appear to be constant and is likely to be dependent on age (Whikehart et al. 2005). Hara and coworkers have isolated corneal endothelial progenitor cells (HCEnPs) and achieved differentiation of these cells into corneal endothelial cells (HCEnCs) with adequate morphology, corneal endothelial marker expression and physiological pump function (Hara et al. 2014).

13.2.4 Characterization of Corneal Stem Cells

13.2.4.1 Characterizing Limbal Epithelial Stem Cells

There is no single specific stem cell marker for LESCs but the expression of a combination of features can be used to characterize this population (Secker and Daniels 2009). This can be partially explained by the persistence of stem cell

markers in the early differentiated phase; cells show an intermediate phenotype until they are fully differentiated and the stem cell markers downregulated (Noisa et al. 2012; Naujok and Lenzen 2012). LESC characteristically lack the expression of a number of differentiation markers, such as the cornea-specific cytokeratin 3 (CK3), cytokeratin 12 (CK12) (Schermer et al. 1986; Kurpakus et al. 1990) connexin 43 and involucrin (Matic et al. 1997; Chen et al. 2004; Shortt et al. 2007a). Pellegrini et al. (2001) have proposed transcription factor p63 as a limbal epithelial stem cell marker. However, basal cells of the peripheral and central cornea can also express p63, so it cannot be used as a specific LESC marker (Chee et al.; Dua et al. 2003; Chen et al. 2004). It has been proposed that the p63 isoform, ΔNp63α is a more specific marker and that cells expressing a high level of ΔNp63 combined with a high nuclear to cytoplasmic ratio are more stem-like than cells expressing lower levels of ΔNp63 (Di Iorio et al. 2005; Arpitha et al. 2005; Priya et al. 2013). In addition to marker expression, organ-specific stem cells can also be identified by the presence of a side population (SP) phenotype. SP cells efflux Hoechst 33324 dye via the ATP-binding cassette transporter subtype G-2 (ABCG2), a multi-drug resistance protein. Therefore ABCG2 has also been proposed as an universal stem cell marker (Goodell et al. 1996; Zhou et al. 2001; Watanabe et al. 2004). Recently *ABCB5*, αvβ5-integrin and the interferon-inducible chemokine CXCL10/IP-10 were also proposed as limbal epithelial stem cell markers (Ordonez et al. 2013; Ksander et al. 2014).

13.2.4.2 Characterizing Mesenchymal Stem Cells

MSCs can be recognized by their characteristic spindle shape and ability to adhere to plastic (Friedenstein et al. 1968). They have been isolated from numerous tissues but as is the case with LESCs, there is a lack of specific markers to characterize the MSCs (Li and Zhao 2014). The ISCT has stipulated minimum criteria that have to be met in order for cells to be identified as MSCs; (i) plastic adherence under standard culture conditions; (ii) positive expression of cell surface markers: CD73, CD90 and CD105 and negative for the expression of CD11b, CD14, CD19, CD79a, CD34, CD45 and HLA-DR and (iii) the capacity for trilineage mesenchymal differentiation: adipogenesis, osteogenesis, and chondrogenesis when using standard in vitro differentiation culture conditions. These criteria were confirmed in corneal cells derived from the stromal periphery and limbus (Dominici et al. 2006; Branch et al. 2012). Characterization of the resulting differentiated keratocytes is based on following molecular markers: keratan sulfate, keratocan and aldehyde dehydrogenase 3A1 (ALDH3A1) (Funderburgh 2000; Du et al. 2005; Pei et al. 2006). It has also been demonstrated that keratocytes express CD133 (Perrella et al. 2007) and do not express ATP-binding cassette transporter subtype G-2 (ABCG2) or *PAX6*. ABCG2 and *PAX6* are frequently used to distinguish keratocyte progenitor cells, which do not express these markers, from the corneal limbal stroma and mesenchymal stem cells that do have a positive expression of these markers (Du et al. 2005; Pinnamaneni and Funderburgh 2012).

13.2.4.3 Charachterizing Corneal Endothelial Stem Cells

Few studies have reported the progenitor capabilities of endothelial cells and even fewer have described the characteristics of these cells other than in telomerase activity. Human corneal endothelial progenitor cells have been characterized using flow cytometry, differentiation and gene expression assays (Hara et al. 2014). Isolation of the endothelial cells with progenitor-like features was performed by selective cell sorting for cell surface marker CD271, which has also been isolated from neural-crest-derived progenitor cells (Morrison et al. 1999). Corneal endothelial cells that expressed CD271 showed the highest proliferative capacity but only when cells were isolated from a young donor (Joyce 2003; Zhu and Joyce 2004; Hara et al. 2014). The progenitor cells also showed positive expression of neural crest markers SOX9 and AP-2β, and also FOXC2 which is necessary in the periocular mesenchyme for the development of the anterior segment of the eye (Gage et al. 2005). CD271 and SOX9 are also instrumental in the development of neural crest cells (Morrison et al. 1999; Cheung and Briscoe 2003).

13.3 Responses to Injury and the Role of Stem Cells in Corneal Repair

Severe trauma to the cornea can result in scarring and blindness if the corneal tissue is not restored appropriately. The commonest causes of corneal injury include infections, thermal, chemical burns and mechanical abrasions, but also hereditary and immune disorders leading to opacification, conjunctivalization, neovascularization, and ulceration. In Sect. 4.5 the clinical applications of stem cells in corneal wound healing will be discussed but first an overview is given of the consequences and the responses to corneal injury. Since the layers of the cornea are so diverse in their embryology, physiological properties and regenerative capacities, the ability of a cornea to recover from injury is dependent on both the surface area of the injury and the depth of the penetration.

13.3.1 Epithelial Repair

In case of a superficial injury only the epithelium is affected. If the epithelial barrier is breached, an immediate physiological, biochemical and anatomical healing response is initiated. Mitochondrial energy production is increased, cell membrane extensions are formed and hemidesmosomal adhesions to the basement membrane are lost (DelMonte and Kim 2011). The proliferative activity in the limbus rises dramatically, up to ninefold, which persists for up to 48 h. During this time, the proliferative activity in the peripheral and central cornea doubles, until wound closure (Cotsarelis et al. 1989; Lavker et al. 1998). This is thought to be the result of

the proliferation of limbal epithelial stem cells and transient multiplication of basal cells (Lehrer et al. 1998; Daniels et al. 2001). The first phase of the response to injury is nonmitotic. The sheet of residual epithelial cells starts migrating and spreading and cells at the advancing edge of a wound extend the sheet to cover the wound (Soong 1987). Matsuda et al. demonstrated that larger epithelial wounds close at a faster rate than smaller ones, with a speed of up to 80 μm per hour (Matsuda et al. 1985). In the second phase, the epithelial cell population is restored by reconstitutive mitosis of the basal cells, the TACs and the limbal stem cells (Wiley et al. 1991). If the limbus is severely damaged and devoid of its stem cell population, this process cannot take place and consequently corneal wounds do not heal adequately.

13.3.1.1 Limbal Epithelial Stem Cell Deficiency

When the source of limbal epithelial stem cells is compromised, the constant regeneration of the corneal epithelial surface is impaired and Limbal Epithelial Stem Cell Deficiency (LSCD) occurs (Secker and Daniels 2009). The cornea is left prone to invasion of conjunctival tissue, neovascularization, chronic inflammation and scarring (Li et al. 2007). Primary causes of LSCD include the partial or complete destruction of the limbus in absence of identifiable external factors (e.g. aniridia, multiple endocrine deficiency associated keratitis, erythrokeratoderma, sclerocornea), whereas secondary causes are mostly external factors that demolish the architecture and the microenvironment of the limbal stem cell niche (e.g. chemical abrasion, thermal burns, Stevens-Johnson syndrome) (Tseng 1989).

Clinically, LSCD can be characterized by a set of symptoms that range from visual impairment to recurrent pain, discomfort, and photophobia. Patients may complain of epiphora, blepharospasm or a red eye (perilimbal vascular injection) (Shapiro et al. 1981; Ahmad 2012). The diagnosis of LSCD can be made clinically in most cases on the basis of history and slit lamp examination. Signs of LSCD include: bulbar/perilimbal vascular injection, loss of limbal anatomy (i.e. loss of Palisades of Vogt), presence of corneal haze, persistent or recurrent epithelial defects, inflammation of the corneal epithelium or stroma, corneal opacification, scar tissue formation, or corneal melting (Baylis et al. 2011). The loss of limbal stem cells through infection, injury or diseases also results in conjuctivalization: the overgrowth of conjunctiva on the cornea. Corneal conjunctivalization can be confirmed using impression cytology to identify goblet cells which, while characteristic of conjunctival epithelium, are typically absent from corneal epithelium. One of the major hallmarks of LSCD is neovascularization.

Corneal neovascularization is the overgrowth of blood vessels from the highly vascular limbal plexus into the cornea,. When the harmony between antiangiogenic and angiogenic factors is disturbed in favour of the angiogenic components, neovascularization occurs. Neovascularization of the cornea has serious implications for the visual acuity and worsens the prognosis of corneal transplantations. It is usually a secondary consequence of trauma, infection, ischemia, chemical abrasions or ther-

mal burns. More than a million people per year lose their vision due to the emergence of new corneal blood vessels (Bachmann et al. 2008). The new vessels cause inflammation, block incoming light and lead to edema. The most important mediator of angiogenesis is vascular endothelial growth factor (VEGF) and therefore anti-VEGF factors (e.g. siRNA, tyrosine kinase, bevacizumab and VEGF trap) are currently under investigation as a potential treatment targets for corneal neovascularization (Chang et al. 2012).

13.3.2 Stromal Repair

The corneal stroma has a highly organized structure and lower density of cells than the epithelium and as such, has a lower capacity for repair and regeneration. Injuries severe enough to breach the epithelium and Bowman's membrane therefore have a higher likelihood of inducing a permanent scar.

Among the commonest causes of stromal injury are infections, thermal, chemical and mechanical abrasions, hereditary and immune disorders. Injury that leads to the loss of specific isoforms of collagen, such as collagen type IV from the epithelial basement membrane, has been associated with an overexpression of matrix metalloproteinases in combination with the activation of inflammatory cytokines. This subsequently leads to the formation of scar tissue and corneal haze (Gabison et al. 2009).

13.3.2.1 Role of MSCs in Stromal Wound Healing

The MSCs respond to corneal injury and inflammation by mobilization, migration and colonization (Kang et al. 2012). The process of stromal wound healing is activated when a corneal injury penetrates the Bowman's or Descemet's membrane and infiltrates the stroma leading to edema and corneal haze. Stromal wound healing involves a variety of growth factors, cytokines and chemokines secreted by the overlying, injured epithelium and keratocytes (Netto et al. 2005). The process is carried out in three phases: (i) repair, (ii) regeneration and (iii) remodelling (Fini and Stramer 2005). Firstly, some of the keratocytes within the area of injury undergo apoptosis, while others are activated and differentiate into a fibroblast-like phenotype (Wilson et al. 1996; Stramer et al. 2003). The new 'repair' fibroblasts can proliferate and synthesize the components required for the formation of new extracellular matrix. After 1–2 weeks, the stroma at the site of injury is invaded by myofibroblasts which promote remodelling by further differentiating into the 'repair' type fibroblasts possessing contractile properties while expressing a wide array of proteins. It has been proposed that the collagen in the stromal wound is reorganized by matrix metalloproteinases (MMPs) which remodel the ECM and the interactions between the matrix and the cells (Maguen et al. 2002; Fini and Stramer 2005; Gabison et al. 2005). The highly light-scattering myofibroblasts de-differentiate

once wound healing is complete and transparency of the cornea should return. If the repair fibroblasts differentiate into scar keratocytes (laying down irregularly spaced collagen) instead of myofibroblasts, they cannot de-differentiate and contribute to a permanent corneal scar (Shah et al. 2008).

13.3.2.2 MSCs Modulate Corneal Angiogenesis

Some in vivo work suggests that applying MSCs on the cornea could inhibit inflammation related angiogenesis after chemical injury (Ma et al. 2006; Oh et al. 2008; Yao et al. 2012), while MSCs in ischemia or tumor models secrete vascular endothelial growth factor (VEGF) and activate angiogenesis (Ball et al. 2007; Beckermann et al. 2008; Tang et al. 2009). In a rat chemical corneal burn model, the expression of antiangiogenic thrombspondin-1 (TSP-1) was upregulated while the proangiogenic factor matrix metalloproteinase-2 (MMP-2), related to inflammation, was downregulated (Oh et al. 2008). This study found no significant differences in VEGF levels for control rats versus MSC-treated rats, while other studies have found a significant downregulation of VEGF expression when MSCs were administered subconjunctivally in the acute phase of a rat alkali burn model (Yao et al. 2012). When human corneal epithelial cells were cocultured with human mesenchymal stem cells, VEGF levels were upregulated and high TSP-1 and MMP-2 levels were expressed. Matrix metalloproteinease-9 (together with MMP-2 and VEGF one of the most important angiogenic factors in the cornea) is normally secreted by human corneal endothelial cells, but the presence of hMSCs has been shown to suppress this secretion (Oh et al. 2009a, b). The antiangiogenic factor TSP-1 inhibits angiogenesis induced by VEGF by suppressing CD36 activation (Cursiefen et al. 2004; Primo et al. 2005; Cursiefen 2007).

13.3.2.3 MSCs Are Effective in Reversing Ongoing Graft Versus Host Disease

Corneal limbal stromal MSCs possess immosuppressive properties, but their potential is significantly weaker than in bone marrow-derived mesenchymal stem cells (Du et al. 2009). MSCs are usually described as MHC I positive and MHC II negative. Mesenchymal stem cells (MSCs) lack the expression of co-stimulatory molecules B71, B72, CD80, CD86, CD40 and CD40 ligand on their surface (Tse et al. 2003; Wong 2011). This phenotype suggests that MSCs may be capable of inducing tolerance, as it is regarded as a hypoimmunogenic phenotype (Javazon et al. 2004). Cells that do not express MHC I, however, are destroyed and therefore the expression of low levels of MHC I is crucial in the protection against natural killer cell-mediated cytotoxicity (Moretta et al. 2001) (see Fig. 13.4). The hypoimmunogenic features of MSCs imply that they can stay under the radar of the immune system and can be used in allogeneic hosts (Di Nicola et al. 2002; Le Blanc et al. 2003a; Ryan et al. 2005). Suppression of activated T cells by MSCs has been shown in vitro,

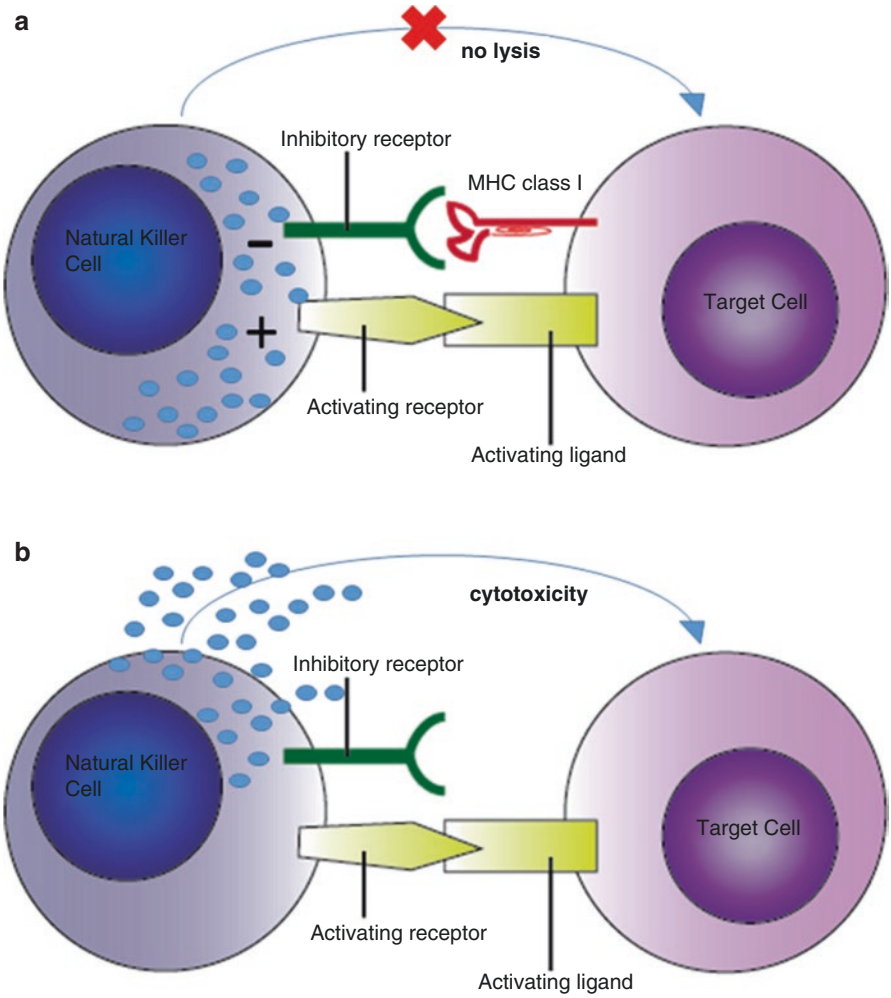

Fig. 13.4 Natural killer cell-mediated cytotoxicity. In presence of MHC class I molecules, granule release is inhibited and the target cell does not undergo lysis (**a**). When the target cells do not present MHC class I molecules, NK cells release cytotoxic granules that will lead to lysis of the target cells (**b**)

regardless of whether the T cells were activated by alloantigens, polyclonal mitogens or CD3 (Di Nicola et al. 2002) and independent of HLA matching between the T cells and MSCs (Le Blanc et al. 2003a). There remains some controversy regarding the mechanism behind this suppression. According to some groups, suppressor activity is exhibited through a soluble factor, whereas other groups claim that direct cell-to-cell contact is an indispensable requirement (Di Nicola et al. 2002; Le Blanc et al. 2003b). The ability to inhibit T cell proliferation with antibodies against hepatocyte growth factor (HGF) and transforming growth factor-β1 (TGF-β1) was

described by Di Nicola et al. in 2002 and in 2012 Garfias and coworkers demonstrated that in the case of limbal MSCs, TGF-β1 induced suppression of T cells (Di Nicola et al. 2002; Garfias et al. 2012). The results suggest that the immunomodulatory properties observed in vitro and the inhibition of proinflammatory immune reactions can be translated to the in vivo situation, but little is known about the host immune response to MSCs. Further claims regarding the in vivo therapeutic potential can only be made after extensive further research into the immunology of MSCs (Javazon et al. 2004).

MSCs can reverse graft-versus-host disease (GvHD) complications (Le Blanc et al. 2004; Ringdén et al. 2006; Le Blanc et al. 2008) and ongoing research will further elucidate the role of MSCs in host-versus-graft disease (HvGD) (Bartholomew et al. 2002; Casiraghi et al. 2008). Even though corneal transplantations are the most successful type of transplantation, allograft failure does occur and the most common cause is irreversible rejection. The immunomodulatory effects of MSCs have been investigated in vivo by transplanting pig corneas into rats and studying the effects of post-operative topical application of allogeneic rat MSCs. Even though IL-6 and IL-10 levels increased significantly, corneal xenograft survival was not prolonged significantly (Oh et al. 2009a). In a subsequent study the researchers made use of a mouse-to-mouse allograft model in which human MSCs were administered preoperatively by intravenous injections. They observed a decrease in surgically induced inflammation, mainly through the secretion of TSG-6 (Tumor necrosis factor-inducible gene 6 protein), and a reduced activation of APCs in the cornea. Allograft survival was prolonged (Oh et al. 2012), and the findings were confirmed by Jia et al. in a rat corneal allograft rejection model, where postoperative instead of preoperative MSC injection was used. Other observations included the reduction of Th1 (type I helper T cell) proinflammatory cytokines, an elevation of IL-4 secretion by T-lymphocytes and an upregulation of Tregs (Jia et al. 2012). It can be concluded therefore, that the suppression of rejection in corneal transplantations is likely dependent on the route and timing of administration of MSCs (Yao and Bai 2013). Watson et al. investigated the therapeutic value of factors secreted by MSCs. MSC-conditioned medium inhibited migration of fibroblasts and their wound healing activities in vitro (Watson et al. 2010). When injected intravenously in an in vivo rabbit alkali burn model MSCs promoted wound healing in synergy with hematopoietic stem cells (Ye et al. 2006) and subconjunctival injection resulted in anti-inflammatory effects (Yao et al. 2012). Systemic administration of MSC in mice led to the homing of MSCs only to the injured cornea, resulting in epithelial regeneration (Lan et al. 2012). Recently, the topical administration of autologous adipose-derived MSCs led to complete epithelial healing in a patient with a persistent post-traumatic sterile corneal epithelial defect, but the mechanisms associated with this process remain unclear (Agorogiannis et al. 2012). Endothelial injuries could also be treated by injection and subsequent homing of MSCs to the injured site, as has been indicated in an human ex vivo corneal wound model (Joyce et al. 2012). Studies focusing on endothelial and epithelial injuries help to elucidate the potential of MSCs.

13.3.2.4 Potential Application of MSCs in Corneal Epithelial Repair

Although the exact mechanisms remain unknown, several studies have reported on the ability of MSCs to make microenvironmental modifications in the cornea. MSCs are relatively easy to isolate and expand, and a number of options have been explored when it comes to administration of MSCs with regards to the cornea, from direct administration to the use of carriers. Recently the potential for transdifferentiation of MSCs into corneal cell types has been explored. Gu et al. have demonstrated both ex vivo and in vivo differentiation of MSCs into corneal epithelial cells in a rabbit model and Reinshagen confirmed the transdifferentiation potential of MSCs in an experimental limbal stem cell deficiency rabbit model (Gu et al. 2009; Reinshagen et al. 2011). Others have confirmed these findings, making use of mice and rat models and even used human MSCs to demonstrate the transdifferentiation potential (Guo et al. 2006; Jiang et al. 2010; Liu et al. 2010, 2012). However, other groups have found that the therapeutic mechanism was not associated with the differentiation, mainly because of a lack of evidence since MSCs and keratocytes have many cell surface markers in common (Ma et al. 2006; Oh et al. 2008). Subconjunctivally injected MSCs did not migrate into the injured cornea nor did they differentiate, indicating that the route of administration is of importance for differentiation into other corneal cell types (Yao et al. 2012).

13.3.3 Endothelial Repair

Endothelial cells do not proliferate in vivo. As the cornea ages, endothelial cells are gradually lost but provided there are sufficient residual cells to compensate, the pump function is preserved. If cells are lost beyond the ability of the residual cells to compensate, the cornea can become edematous. The endothelium is susceptible to all kinds of stress, whether it is metabolic, toxic or caused by alterations in pH. Some contact lenses have shown to cause hypoxic stress that affects the endothelium and leads to an impaired functioning of this delicate layer (Beuerman and Rozsa 1985; Polse et al. 1990). Another risk factor is hyperglycemia, it has been demonstrated that diabetes has an influence on the cell density and the level of polymegathism and pleomorphism (Roszkowska et al. 1999).

There are two possible healing pathways following endothelial injury; a regenerative and a non-regenerative (or fibrotic) option. The first mechanism occurs mainly following a minor injury and provokes the remaining cells at the site of the injury to increase in size in order to cover the wounded area. This state is also known as polymegathism and is the first stage of regenerative endothelial wound healing. A barrier is restored, albeit with incomplete tight junctions and a limited number of pump sites. In the next phase the barrier is restored. The enlarged cells lose their hexagonal shape and become polymorphic, and the tight junctions and pump sites return to a level that approaches the original state. In the final stage, which can take up to several months, the hexagonal shape of the cells is restored

(Watsky et al. 1989; Peh et al. 2011). Though limited pump function may be restored, the endothelium cannot return to the level of its previous uninjured state. Following major insult to the endothelium, a non-regenerative pathway is activated. The endothelial cells lose their shape and the monolayer is transformed into a multilayer of fibroblastic cells due to the downregulation of N-Cadherin. The fibroblasts deposit ECM onto the Descemet's membrane in a process known as endothelial-to-mesenchymal transition (EnMT). An additional retrocorneal fibrous membrane may be formed in between the endothelium and Descemet's membrane by the expression of collagen type I instead of the resident collagen type IV (Lee and Kay 2006; Li et al. 2013). Severe damage to the endothelium may result in irreversible changes to the cytoskeleton and considerable cell loss (Kim et al. 1992). Once the threshold for endothelial cell loss is met, i.e. that more than 75 % of the cells are lost or if less than 500 cells/mm^2 remain, corneal edema occurs leading to opacification (Polse et al. 1990). The recent advances in human corneal endothelial progenitor cell characterization, opens up the possibilities for future treatments in regenerative medicine. Various attempts with differing culture and transplantation techniques remain unsatisfactory in treating endothelial dysfunction as none of them lead to the regeneration of the endothelium (Ishino et al. 2004; Sumide et al. 2006; Mimura et al. 2008; Watanabe et al. 2011). Hara and coworkers, however, did successfully transplant a tissue-engineered human corneal endothelial progenitor cell sheet into a rabbit eye with corneal endothelial deficiency and observed increased transparency and a reduction in corneal thickness (Hara et al. 2014).

13.4 Traditional Strategies for Corneal Reconstruction

13.4.1 Ocular Surface Reconstruction in Limbal Stem Cell Deficiency

The use of autografts and allografts was the first proposed treatment option for the ocular surface disorders or traumas characterized by the absence or damage to the limbus. In patients with severe limbal damage, a corneal transplant is not likely to survive because a healthy epithelium is the key factor. Re-establishing healthy limbal function therefore is often the first step in restoring vision following severe anterior segment trauma (see Fig. 13.5). Rivaud and coworkers (1986) were the first to include a piece of the limbus in a conjunctival autograft, with relatively high success rates, since prior to that only the conjunctiva was grafted (Vorkas 1981; Kenyon et al. 1985), in a method first described by Thoft (1977, 1979). Tseng made a distinction between conjunctival and limbal grafting and concluded that only in the case of limbal grafting the corneal epithelial phenotype was restored, as opposed to conjunctivalization of the cornea when only bulbar conjunctiva was used (Tseng 1989). In the same year they performed limbal transplantations on two patients; the first case received an ipsilateral autograft, and the second a contralateral autograft

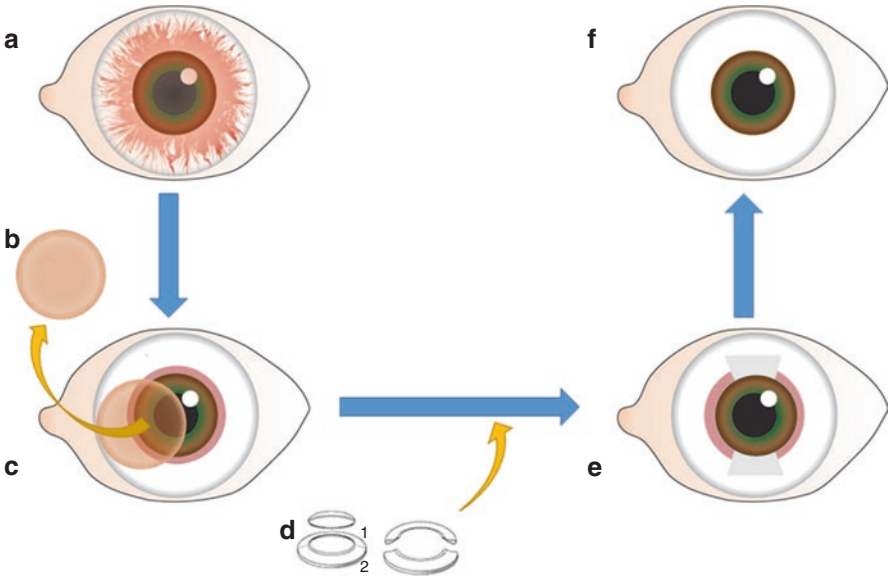

Fig. 13.5 Patient with LSCD and heavily vascularized cornea (**a**). Removal of fibrovascular pannus tissue until the limbus (**b**) exposes the non-vascularized deeper layers of the cornea (**c**). Depending on the health of the patient's contralateral eye, conjunctival-limbal autografts are harvested and placed at 12 and 6 o'clock (**e**) or the limbus is replaced entirely by two 180° strips of allogeneic donor tissue (KLAL) (**d**). The transplanted limbal tissue restores the epithelial stem cells and limbal barrier and generates healthy, transparent and non-vascularised corneal epithelium (**f**)

with a subsequent keratoplasty (Kenyon and Tseng 1989). Both patients achieved encouraging results.

The feasibility of a limbal autograft in the case of a unilateral condition is still one of the major assets, however there are some important risks to consider. A large biopsy is required (minimally 60° of the limbus) and, as such, there is risk of inducing LSCD in the donor eye during tissue harvest. An alternative option, the keratolimbal allograft (KLAL), obtains limbal graft tissue from cadaveric donors. The high rate of rejection and the severity of side effects from immunosuppressants that are required with allogeneic transplantations (Secker and Daniels 2009) is a major disadvantage of this approach. Good long-term clinical outcomes have been shown for limbal allografts, but there is no proof of persistence of epithelial donor cells on the surface of the recipient's cornea. It is hypothesized that the transplanted microenvironment is adequate to sustain the remaining host stem cells, which wouldn't be able to survive otherwise, by supplying growth factors to the stem cell niche (Williams et al. 1995; Henderson et al. 1997, 2001). This is an important observation with regards to future developments in corneal tissue engineering; if the provided scaffold is suitable, the host cells could find their way into the scaffold, without the need for donor or host cells to be included as part of the transplanted graft. This mechanism would obviously require some residual host stem cells at the limbus, which is not always the case.

13.4.2 Transplantation Options for the Corneal Stroma

In addition to treating the limbus and epithelium, there are also different treatments available for stromal defects (Fig. 13.6 B, C and D). Traditionally, cadaveric corneal donor tissue has been used to replace damaged stromal tissue in a Penetrating Keratoplasty (PK) surgery. Penetrating keratoplasty is the traditional transplantation in which all three corneal layers, epithelium, stroma and endothelium are replaced by donor tissue. Newer developments in stromal surgery include Anterior Lamellar Keratoplasty (ALK) and Deep Anterior Lamellar Keratoplasty (DALK) but both of these techniques still require cadaveric donor tissue which is limited globally. The main difference between (D)ALK and PK is that in (D)ALK, being a partial thickness stromal replacement procedure, the Descemet's membrane and the underlying endothelium remain untouched and there is no penetration into the anterior chamber. Preserving the endothelium reduces the risk of endothelial rejection and chronic cell loss when compared with PK (Borderie et al. 2009; Reinhart et al. 2011) (Sugita and Kondo 1997; Shimazaki et al. 2002b; Armitage et al. 2003). The advantages of anterior lamellar keratoplasty over penetrating keratoplasty are apparent, but the visual acuity of the recipients of lamellar grafts may be impaired by host-donor interface irregularities (Saini et al. 2003; Funnell et al. 2006). Other studies have, however reported comparable optical performance outcomes (Panda et al. 1999; Watson et al. 2004).

The function and morphology of the epithelium undergo changes after penetrating keratoplasty, such as increased cell area and altered cellular metabolism (Vannas et al. 1987; Feiz et al. 2001). A marked decrease in the number of cells (epithelial, keratocytes and endothelial cells) in corneal grafts after transplantation has been described (Imre et al. 2005; Niederer et al. 2007). It remains unclear why the density of keratocytes in the donor cornea decreases after transplantation (Bourne 2001; Imre et al. 2005). One possibility is that the keratocytes undergo increased apoptosis, which has been noted in particular at the wound edges of transplanted corneas (Ohno et al. 2002). Another option is that the exact number of keratocytes is not reduced, but that due to corneal swelling, the density of distribution is lowered (Niederer et al. 2007). Nevertheless, it has been proven that donor cells are gradually replaced by the host's own keratocytes, with the exception of small populations of donor cells that can persist for up to 5 years (Hanna and Irwin 1962; Wollensak and Green 1999; Hori and Streilein 2001).

13.4.3 Corneal Endothelial Transplantation

Endothelial defects can be treated by performing Descemet's Membrane Endothelial Keratoplasty (DMEK) and Descemet's Stripping Endothelial Keratoplasty (DSEK), where the patient's own endothelium is removed and replaced with a layer derived from a cadaveric donor (Fig. 13.6 E and F). Descemet stripping endothelial

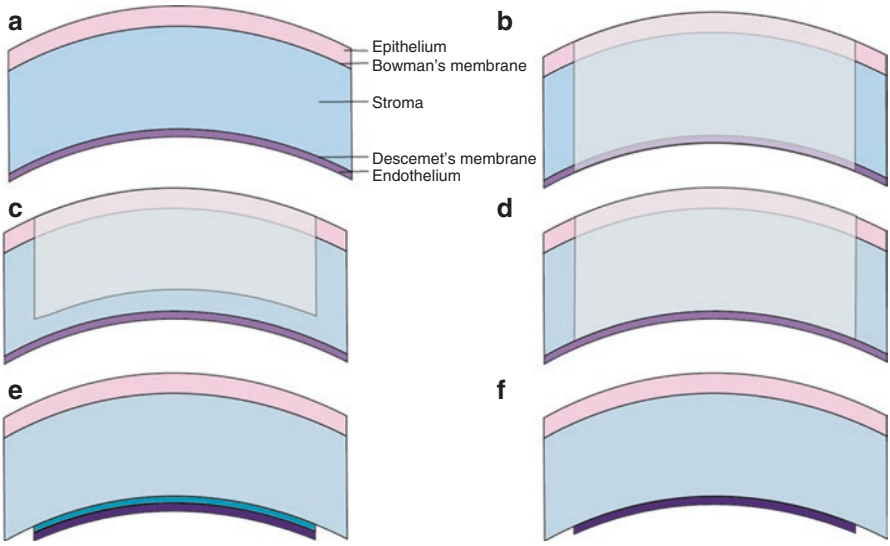

Fig. 13.6 Tranplantation options for stromal and endothelial deficiencies. The five layers of the cornea; the epithelium and stroma are separated from each other by Bowman's membrane, whereas stroma and endothelium are separated by Descemet's membrane (**a**). Penetrating keratoplasty or PK; epithelium, stroma and endothelium are replaced by donor tissue (*grey insert*) (**b**). Anterior lamellar keratoplasty or ALK; only epithelium and the affected part of the stroma are replaced by donor tissue (**c**). In the case of DALK, the full thickness of the stroma is replaced together with the epithelium (**d**). In Descemet's stripping (automated) endothelial keratoplasty (DS(A)EK) only the endothelium is replaced by cadaveric endothelium (*dark purple*) and stromal (*darker blue*) donor tissue (**e**). In Descemet's membrane endothelial keratoplasty (DMEK) only the endothelium is replaced by donor tissue (**f**)

keratoplasty (DSEK) is a technique where the corneal endothelium, Descemet's membrane and thin layer of stroma are isolated from a cadaveric donor cornea and introduced into the diseased cornea after removal of the diseased layers. The donor material is pressed against the posterior surface of the stroma using an air bubble to improve graft adhesion. With the implementation of a microkeratome, graft isolation is more standardized and thinner donor grafts can be achieved. This modification is known as Descemet Stripping Automated Endothelial Keratoplasty (DSAEK) (Maier et al. 2013). In DMEK surgery only the donor endothelium with Descemet's membrane is transplanted without any residual stroma (Melles et al. 2006). The Descemet's membrane is manually peeled off together with the endothelium and automatically forms a roll with the endothelium on the outside when placed in a fluid. This "DMEK roll" is inserted in the anterior chamber, unrolled and positioned with the help of an injected air bubble.

DMEK is preferred over DSAEK because it results in a better vision, a more rapid recovery and less rejection of the graft. The reason for this improved outcome is that the diseased endothelium is replaced in an anatomically correct manner, as the graft is only 10–15 μm thick and (almost) no stroma is attached to the

endothelial graft. The incision needed for this surgery is also very small, causing no or only a small change in refraction. The fact that there is less tissue transplanted compared to DSAEK, additionally reduces the risk of graft rejection. DSAEK is a standardized procedure, while DMEKs cannot yet be reproduced by all surgeons. DMEK does however have a flat learning curve and a higher rate of postoperative re-intervention.

13.5 Tissue Engineering for Corneal Reconstruction

The need for tissue-engineered alternatives to replace donor-derived epithelium, stroma and endothelium remains high. Transparency and tissue strength are two essential properties to take into account when considering corneal replacements. The natural structure is difficult to reproduce, since the strength and transparency of the tissue are characteristics inherent to the intricate architecture of the cornea. Similarly another vital challenge is biocompatibility, as it is crucial that tissue-engineered constructs are well-retained in the eye. Currently, there are two approaches to corneal tissue engineering; a cell-based approach and a scaffold-based approach. In cell-based approaches, the cells themselves and the information that lies within the cells are the most important contributors to the tissue engineered construct. This approach is most often used with regards to regeneration of the epithelium and the endothelium, since these layers are closely associated with their basal layers in the cornea in vivo. Scaffold-based approaches are more focused on the development and optimization of new substrates to mimic the corneal stroma.

13.5.1 Cell-Based Approach to Tissue Engineering

In recent years the focus of cell-based tissue engineering has been on the corneal epithelial tissue engineering, possibly because it is the most superficial layer and easily accessible. Many attempts have been made at generating a sheet of epithelial cells that is readily transplantable without the need of sutures (Yamato and Okano 2004; Nishida et al. 2004). After transplantation, the cell sheet should attach to the remaining native ECM in recipient eye. Cell sheets can be grown from a small biopsy of healthy epithelial tissue containing limbal epithelial stem cells, and this ex vivo expansion is an enormous advantage over the transplantation of large pieces of limbus from a healthy to a diseased eye, reducing the risk to the healthy donor. The use of laboratory-expanded limbal epithelial cells was proposed by Lindberg and coworkers in 1993 and first performed on patients by Pellegrini and collaborators in 1997 (Lindberg et al. 1993; Pellegrini et al. 1997). Since the first cultivated limbal epithelial transplantations (CLET), a lot of research has been done and more recently a number of clinical trials have been published focussing on the use of

in vitro expanded LESC (Tsai et al. 2000; Koizumi et al. 2001; Grueterich et al. 2002; Shortt et al. 2007b).

13.5.1.1 Epithelial Tissue Engineering

A variety of carrier layers have been used, from amniotic membranes (Shimazaki et al. 2002a; Zakaria et al. 2010), polystyrene (Feng et al. 2014), nanofiber scaffolds (Zajicova et al. 2010), siloxane-hydrogel contact lenses (Di Girolamo et al. 2007), silk fibroin (Bray et al. 2011), chitosan (Grolik et al. 2012), keratin films (Reichl et al. 2011), to fibrin (Rama et al. 2001). Depending on the research group, limbal epithelial cells were cultured with murine-derived 3T3 fibroblast feeder cells or in xenobiotic-free culture systems. The biological mechanisms behind the potency and effectiveness of in vitro expanded LESC remain somewhat unclear, but nonetheless the clinical results are promising (Baylis et al. 2011; O'Callaghan and Daniels 2011; Basu et al. 2012; Menzel-Severing et al. 2013). The most commonly used carriers are biodegradable fibrin layers or thermo-responsive polymers. The use of fibrin carriers was first described in 2007 and it was shown that the fibrin layer can be digested once the cells have formed stratified, functional multilayer sheets (Higa et al. 2007). In 2015 the first advanced therapy medicinal product (ATMP) containing corneal stem cells was approved by the European Medicines Agency. The product, named Holoclar, is obtained through cultivation of an autologous limbal biopsy on fibrin using mouse 3T3 fibroblasts and fetal bovine serum (FBS) (Rama et al. 2010). The long term results are encouraging however the number of patients that can be treated with the product is limited. Holoclar is currently indicated for unilateral chemical or thermal burns with limited to no stromal involvement, which is the minority of limbal stem cell failure cases. More recently, the use of xenobiotic-free limbal epithelial stem cell cultures on human amniotic membranes is currently being investigated in a phase II multicenter clinical trial. This method could allow the use of allogeneic donor material in cases of bilateral LSCD and may provide an option for patients bilateral disease and stromal involvement (Zakaria et al. 2014). PNIPAM (poly(N-isopropylacrylamide)) is another alterative material that is hydrophobic at 37 °C and facilitates attachment, proliferation and secretion of ECM by the expanded cells. When the temperature is lowered to 20 °C the polymer shifts to a hydrophilic state and starts expanding. This expansion leads to cellular detachment and an intact functional sheet of epithelial cells is obtained. The main advantage over proteolytic cell harvesting methods is that the cell-cell contact is maintained along with its ECM (Yang et al. 2006; Hayashida et al. 2006).

In the case of bilateral corneal blindness, there is no healthy limbal tissue left for autografting or explanting. Treatment options for these patients were previously limited to allografts from relatives or from cadaveric donor cornea. Such treatments, however, came with an additional risk of disease transmission and graft failure due to rejection. The use of systemic immunosuppressants to prevent graft rejection in some cases increased the risk of developing malignant tumors (Fernandes et al.

2004; Holan and Javorkova 2013). These risks in combination with the severe shortage in donor corneas, have led to a number of alternative stem cell sources, mainly non-ocular, being proposed as alternative treatment options for the regeneration of corneal epithelium. Most of these cells are easily accessible and available in large quantities. Not all of these alternative stem cell sources for corneal regeneration have been used in a clinical setting yet, but due to their unique properties they are seen as candidate cell sources for future clinical applications, also for stromal and endothelial treatments.

Induced Pluripotent Stem Cells

Induced Pluripotent Stem Cells (iPSCs) are dedifferentiated adult cells. The technique was first described in 2006 using specific transcription factors to induce their de-differentiation (Takahashi and Yamanaka 2006). In 2012 corneal epithelial cells were differentiated from iPSCs (from dedifferentiated corneal limbal epithelium) (Hayashi et al. 2012). They made a comparison with iPSCs from dermal fibroblasts and found that higher levels of specific corneal epithelial differentiation markers were expressed than when iPSCs from dermal fibroblasts were used. One of the major drawbacks is that the limbal epithelial iPSCs can also differentiate into other cell types, which is to be avoided in order for iPSCs to be used in the regeneration of corneal epithelium.

Mesenchymal Stem Cells from Non-ocular Tissue

Mesenchymal stem cells can be derived from other tissues as well. The most investigated sources of MSCs are the bone marrow and adipose tissue. These MSC (like the MSC in the cornea) are multipotent, express the characteristic MSC cell surface markers and are plastic adherent (Pittenger et al. 1999). They can differentiate into corneal epithelial cells (by making use of co-culturing techniques and preconditioned media) (Gu et al. 2009) and in earlier animal model studies where the cells did not differentiate, it was seen that the corneal damage was nonetheless mitigated by the anti-inflammatory and anti-angiogenic attributes of the MSCs grown on human amniotic membranes (Ma et al. 2006).

Conjunctival Epithelial Stem Cells

Conjunctival epithelium and goblet cells are derived from a common progenitor. These progenitor cells have a high proliferative capacity and, at least twice each lifetime, give rise to goblet cells (Pellegrini et al. 1999). The expression of cell markers in the basal conjunctival epithelium is similar to that in the corneal epithelium (Qi et al. 2010). Ocular surface damage has been treated successfully in patients by making use of cultured conjunctival epithelial stem cells (Sangwan et al.

2003). Unfortunately in most LSCD patients the conjunctiva is also affected, making it unsuitable for cell culture and transplantation.

Oral Muscosal Epithelium

Oral mucosal stem cells express limbal stem cell markers and have the ability to be reprogrammed into corneal epithelium-like cells (Nakamura et al. 2007). Oral mucosal epithelial cells have been engrafted onto the ocular surface in patients after alkali burns (Inatomi et al. 2006). Cultured autologous oral mucosal epithelial cell sheets (CAOMECS) are bioadhesive and thus can be grafted without the need of sutures and have been proposed as an alternative to the use of an allogeneic donor in the case of total bilateral LSCD. Results from clinical trials are however not always successful, with some patients having severe adverse reactions to the grafts (Satake et al. 2011) while for others this has been proven to be a successful treatment method (Inatomi et al. 2006; Burillon et al. 2012; Kolli et al. 2014).

Dental Pulp Stem Cells

Immature stem cells can be isolated from the dental pulp of human deciduous teeth. These human immature dental pulp stem cells (hIDPSCs) show expression of both human embryonic and mesenchymal stem cell markers. They can also undergo trilineage differentiation, which is a requirement set by the ISCT for mesenchymal stem cells (Kerkis et al. 2006). In a more recent study by the same group it was discovered that hIDPSCs also express limbal epithelial stem cell markers and that they are a treatment option for total LSCD in rabbits (Monteiro et al. 2009). So far, no clinical trial has used these cells in human patients but very recently it has been shown that also adult human dental pulp stem cells (DPCs) produce corneal stromal extracellular matrix containing human type I collagen and keratocan when injected into a mouse model (Syed-Picard et al. 2015).

Hair Follicle Stem Cells

Hair follicles contain stem cells of epithelial origin in the outer root sheath and mesenchymal stem cells in the dermal papilla and the connective tissue sheath. Since this discovery several research groups have targeted the human hair follicle as a resource for stem cells (Cotsarelis et al. 1990; Yu et al. 2006; Meyer-Blazejewska et al. 2012; Yang and Xu 2013). Hair follicle stem cells can be reprogrammed to form corneal epithelial cells when cultured in conditioned media (Blazejewska et al. 2009) and can terminally differentiate into a corneal epithelial-like phenotype when transplanted in vivo (Meyer-Blazejewska et al. 2012). No clinical trials have examined the potential of hair follicle stem cells in human patients.

Amniotic Epithelial Cells

With low immunogenicity, production of epithelial growth factors and the ability to transdifferentiate (with CK3/12 expression in terminally differentiated cells) human amniotic epithelial cells (HAECs) are a hightly suitable candidate for corneal reconstruction (Miki et al. 2005; Pratama et al. 2011; Zhou et al. 2013). This has been demonstrated in rabbit models (Liu et al. 2013; Zhou et al. 2014). Differentiated cells were anatomically and physiologically similar to corneal epithelial cells and the future prospects for this cell type looks promising (Fatimah et al. 2010; Pratama et al. 2011).

13.5.1.2 Corneal Endothelial Regeneration

Corneal endothelial cell sheets have been produced using comparable methods as for epithelial cell sheets. Currently, two different approaches are being considered for the regeneration of the human corneal endothelium (see Fig. 13.7). The first approach is to inject ex vivo expanded HCEnC into the anterior chamber of the eye. The efficacy of this technique has already been determined in rabbit and monkey models (Mimura et al. 2004; Okumura et al. 2009). Kinoshita et al. are the first research group to enter human clinical trials with an injection based cell therapy to treat corneal endotheliopathies. The concept is based on the capacity of the HCEnC to attach and form a new corneal endothelial barrier following injection of endothelial cell suspension into the anterior chamber. Positioning the patient face down for a few hours would allow the cells to settle and attach to the resident Decemet's membrane, thereby potentially reforming the barrier but until now this has only been validated in animal models.

The second option is to design corneal endothelial cell grafts by expanding HCEnC in culture and seeding them onto an appropriate cell carrier. These composite grafts mimic current corneal endothelial grafts and could overcome the global shortage of donor corneas used for transplantation. Still, standardized cell culture protocols are lacking and there is no consensus yet on the perfect cell scaffold, with different options that extend from natural grown membranes (e.g. amniotic membrane, lens capsule etc.) to biological carriers (e.g. collagen based sheets) or synthetic polymers (e.g. Thermo-reversible Gelation Polymers) (Teichmann et al. 2013). Therefore this method to treat corneal endotheliopathy is still under development and limited to preclinical studies in animal models (Ishino et al. 2004; Mimura et al. 2004; Yoeruek et al. 2009; Ju et al. 2012; Hara et al. 2014).

Endothelial cell culture techniques involve use of conditioned medium from human bone marrow–derived mesenchymal stem cells to enhance HCEnC proliferation through the PI3-kinase and ERK1/2 pathways which results in degradation of p27, a cyclin dependent kinase inhibitor that arrests HCEnC in G1 growth phase (Polyak et al. 1994; Nakahara et al. 2013). Additionally, Y-27632, a Rho-associated kinase (ROCK) inhibitor, is not only used to increase proliferation, but also attachment of HCEnC. This ROCK-inhibitor mediates proliferation by

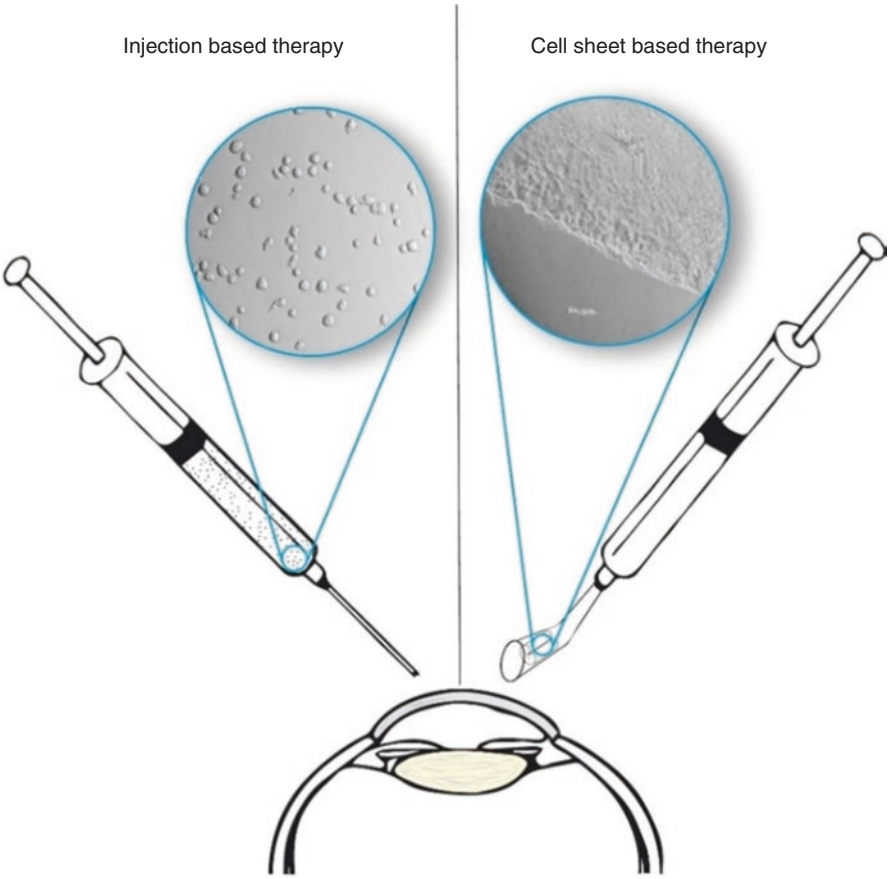

Fig. 13.7 Two different approaches to corneal endothelial tissue engineering. First approach (*left*) is based on the injection of a suspension of HCEnC into the anterior chamber. In the second approach (*right*) an engineered cell sheet with HCEnC is transplanted as a roll, which unfolds and attaches to the posterior surface of the cornea once inserted

inducing G1 to S phase transition via upregulation of cyclin D and a decrease of p27. Furthermore, TGF-β inhibitors are applied to avoid the expected endothelial to mesenchymal transition that is observed in long-term cultivation of HCEnC (Okumura et al. 2013).

Even though the injection-based method has progressed to human clinical testing, there is no absolute preference for one of these methods (scaffold based tissue engineered products versus cell based injections) as both have pros and cons. Introducing a composite graft in the eye is more invasive and could influence visual acuity more than injection therapy. On the other hand injected cells free floating in the anterior aqueous, could cause elevated intraocular pressure due to blockage of Schlemm's canal or be flushed away with the aqueous flux present in the anterior chamber (Okumura et al. 2014).

13.5.2 Scaffold-Based Approach: Stromal Tissue Engineering

Many different biomaterials have been the subject of repeated attempts to recreate layers of the cornea. Scaffold-based corneal tissue engineering focuses mostly on developing a substitute for the natural corneal stroma. There are two general lines in the development of scaffolds for corneal tissue engineering; those using collagen-based scaffolds with crosslinkers, and those using non-collagen synthetic materials. Collagen types I and III have been the focus of many studies (Merrett et al. 2008) though synthetic polymers are also popular, as their mechanical properties are promising (Hu et al. 2005; Zorlutuna et al. 2006). Collagen gels degrade quickly in vivo and do not have the intrinsic strength observed in some synthetic polymers. Different crosslinkers have been tested to increase the stability of the collagen scaffolds. Glutaraldehyde is frequently used as a crosslinker, but its cytotoxic effects are a major disadvantage (Doillon et al. 2003). Polypropyleneimine octamine dendrimers have also been used, because the dendrimers increase the number of amine groups that react with carboxylic groups in the crosslinking process. A third common crosslinking option is 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC) (Fagerholm et al. 2010). Gels crosslinked with octamine dendrimers have been proven to be mechanically superior to both EDC and glutaraldehyde at different concentrations of collagen. The suture strength was much lower than in the natural cornea however and this is a problem which must be resolved prior to use in clinical applications (Duan and Sheardown 2006). Apart from collagen gels, sponges and films have been tested in corneal tissue engineering. Both tissue transparency and mechanical properties have been demonstrated to be superior in collagen sponges as opposed to gels (Orwin et al. 2003; Borene et al. 2004).

Griffith et al. were the first to transplant collagen scaffolds as deep anterior lamellar grafts in a phase I clinical trial in ten patients. They observed a regeneration of the epithelium in all ten patients, as well as subepithelial nerves and growth of stromal cells into the implanted scaffolds after 9 months (Fagerholm et al. 2009; Griffith et al. 2009). Two follow-up studies, one after 24 months and one after 4 years, revealed that in each of the patients, the implant remained stable and avascular, the tear film was restored and further recruitment of stromal cells into the implanted scaffolds occurred. No infections occurred, even without long-term steroid treatments that are required in donor cornea patients (Fagerholm et al. 2010). These results suggest that as long as healthy stem cells are present in the host bed, tissue-engineered scaffolds can be cell-free implants. The potential of corneal MSCs to differentiate makes them excellent candidates for the development of a tissue-engineered corneal stroma (West-Mays and Dwivedi 2006). Stromal fibroblasts can be induced to secrete extracellular matrix, to construct lab grown stromal matrices. The use of ascorbic acid in the culture increases secretion of collagen and proliferation of the fibroblasts (Saika et al. 1992). In a study by Guo et al. (2007), the fibroblasts formed layers of collagen fibrils similar to the native state, albeit slightly thicker than natural cornea. These structures are limited by size and additional research may determine which factors influence fibril diameters (Guo et al. 2007).

Cells that are grown on matrices to which a unidirectional stress is applied, can align along the axis of the stress and also deposit ECM in an aligned fashion (Karamichos et al. 2007). Corneal fibroblasts produce more collagen in constrained circumstances, suggesting the need for some kind of mechanical stimulus. In recent years, bioreactors have been used to implement this mechanical signaling in order to develop natural cornea-like tissue engineered constructs (Cahill et al. 2005; Orwin et al. 2007; Leonard et al. 2012). Basu et al. have demonstrated the differentiation of stromal cells into functional keratocytes in vitro in the presence of autologuous serum and further investigated the in vivo potential for corneal repair. Human limbal biopsy-derived stromal cells were embedded in fibrin gel and applied to the surface of a corneal debridement wound in a murine model upon which the damaged stromal tissue was regenerated, resulting in stromal matrix indistinguishable from the native corneal stromal matrix (Basu et al. 2014). The potential of this autologuous cell-based treatment is now under investigation as a clinical trial has been set up at the L V Prasad Eye Institute, India.

13.6 Conclusion

The ability to see our surroundings is possibly the most invaluable of senses in human life. Each year hundreds of thousands of new cases of corneal visual impairment occur, mostly caused by systemic diseases, inflammation and trauma to the eye with subsequent scarring. Loss of vision leads to a significant reduction in the quality of life and unfortunately many of the conditions that cause visual impairment are notoriously difficult to treat. Recently a number of new approaches have been explored, including the use of stem cell technologies as a treatment for different types of visual impairments. The cornea is comprised of a variety of different stem cell types, each having specific characteristics that are used to distinguish it. Injuries to the cornea elicit a number of responses for which specific treatments are required, and corneal stem cells possess wound healing capacities which make them suitable candidates to enhance current treatments.

In the past two decades a lot of progress has been made in the field, establishing the therapeutic potential of corneal stem cells: corneal stem cells in different layers of the cornea were identified and characterized, the in vitro cultivation potential was demonstrated, confirming function of the stem cells, followed by insight into the regenerative capacity of these stem cells. The role of stromal mesenchymal and limbal epithelial stem cells in the cornea and their therapeutic potential is starting to gain importance as research advances. The recent isolation of corneal endothelial progenitor cells is an important step towards the treatment of endothelial deficiencies. Alongside these corneal stem cell sources, other sources are being explored continuously, leading to new approaches and the development of novel therapies. Restoring the transparency of a diseased cornea remains a key feature in corneal stem cell research and as research in regenerative medicine and more specifically tissue engineering advances, a promising future lies ahead.

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