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Bone Marrow Stem Cell Therapy for Stroke

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To our families

Preface

The book *Bone Marrow Stem Cell Therapy for Stroke* has been created to be the primary resource for scientists, clinicians, teachers, students, and the public at large in the area of cell therapy for stroke. I am honored to have had the opportunity to edit the book with our coeditors, Qichuan Zhuge and Xunming Ji. The contributions of the editors and editorial broad cannot be overestimated.

We know that stroke remains the fifth leading cause of death, with an incidence of ~700,000 events per year and a prevalence of about 4.8 million individuals, of which about 80 % are ischemic and 20 % are hemorrhagic. About one-third of these people fail to survive the event, and of the survivors, 90 % suffer permanent deficits. The most recent major advance in treatment, the use of thrombolytic agents to dissolve clots, appears to be effective only within about the first 3 hours after the initial onset of symptoms. Widely effective treatments for stroke remain elusive, and no effective treatment is available for chronic stroke except a lengthy program of rehabilitation, all the more emphasizing the need for new therapeutic developments. Stem cell transplantation offers an exciting new therapeutic avenue for stroke, because stem cell not only prevent damage, but also actually repair the injured brain.

Many studies have demonstrated favorable results in animal models with various stem cells including bone marrow-derived stem cells (BMSCs), which consequently have resulted in several early Phase I and II clinical trials with promising outcomes. In addition, BMSCs are easy to obtain and expand in culture; using the patient's own BMSCs would eliminate the risk of rejection, and BMSCs have the capacity to migrate to the injury site, permitting systemic administration. This book reviews recent advances in all aspects of BMSC-mediated stroke treatment in animal models of stroke and clinical trials.

The book is organized into 14 major areas, starting with an introduction of stroke pathophysiology and cell therapy, followed by BMSCs characterization, isolation, culture, and identification. The third and forth chapters cover the topics of mobilization and homing of bone marrow stem cells and interaction of BMSCs with other cells. The next three chapters deal with the various types of BMSCs in stroke treatment and co-transplantation strategies and combination therapies for stroke.

The tenth section reviews the clinical studies of BMSC therapy in stroke patients. The 11th chapter is dedicated to the area of the optimal condition of transplantation for stroke treatment. The 12th and 13th chapters summarize the potential mechanisms of transplanted cell-mediated recovery after stroke. The last section of the book is dedicated to imaging and tracking transplanted BMSCs after stroke.

I thank our colleagues, who have contributed their expert advice to the preparation of the first book to focus on BMSC therapy for stroke. I am especially indebted to the staff at Springer Publishing, who have been enormously helpful in moving this book through editing and production. I hope our efforts will help to demystify stem cell therapy for stroke for researchers and clinicians and contribute to provide stroke patients better and more effective treatment.

Fort Worth, TX, USA Kunlin Jin
September 8, 2016

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Chapter 1
Ischemic Stroke Pathophysiology and Cell Therapy

Changhong Ren, Rongrong Han, Jingfei Shi, and Xunming Ji

Abstract Current evidence shows great promise for stem cell transplantation as a new therapeutic strategy for stroke. However, stem cell transplantation for stroke is still in its infancy, with many issues that need to be addressed in order to achieve the full potential of stem cell therapy for stroke. Among the major hurdles for successful clinical translation is determining the therapeutic time window, stem cell type selection, delivery route, and underlying cellular and molecular mechanisms. In this chapter, we attempt to review the basic knowledge of pathophysiology and summarize the different stem cells for stroke treatment.

Keywords Stroke • Ischemia • Transplantation

1.1 Introduction

Stroke remains a worldwide health burden, causing high morbidity, mortality, and costs to health care [51]. It is the foremost cause of long-term disability in the USA and has a high cost to society totaling $38.6 billion in 2009 [65]. Among the various types of strokes, patients are most commonly seen with ischemic stroke, with about

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80 % and over being stricken with this specific disease. Presently, the only FDA-approved treatment is thrombolysis by way of tPA administration within a narrow 4.5 h therapeutic window after stroke onset [40]. Because of its short treatment window and the accompanying concern of hemorrhage when given outside this window, thrombolytic therapy is thus not widely used [123]. About 4 % of stroke patients have been seen to benefit from reperfusion therapies, but only a dismal 10 % of stroke survivors could return to living on their own. In this regard, a huge challenge is posed to researchers and clinical investigators to develop other restorative therapies for the treatment of ischemic stroke. Mechanistic studies of neuronal cell death have resulted in several treatment plans that seek to impede secondary biochemical changes, which consequently decrease the size of brain damage stemming from cerebral ischemia. Incidentally, these mechanisms underlying disproportionate neuronal death in ischemia could be relevant to other neurodegenerative diseases facing the same issue.

This chapter seeks to summarize several mechanisms that have been shown to worsen neuronal death in cerebral ischemia as well as provide a succinct review of current transplanted cell types and their efficacies in the clinic.

1.2 Pathophysiology of Ischemic Stroke

Although there are many etiologic mechanisms, the common pathway of ischemic stroke is lack of sufficient blood flow to perfuse cerebral tissue. Interruption of forward blood flow at any point can lead to irreversible neuronal damage. Ischemic stroke is caused by either of the three main mechanisms, namely, thrombosis, embolism, or global ischemia. Of course, there is no one mechanism that can account for all ischemic strokes. In actuality, the number of factors responsible for uncommon stroke syndromes are numerous. However, vasospastic strokes and a form of arterial inflammation are the more prominent infrequent causes of stroke.

1.2.1 Thrombosis

In situ thrombosis is the formation of a clot in an artery that persists long enough to cause ischemic insult to the cerebral tissue supplied by the affected vessel. Thrombosis is often triggered by pathology in the local endothelium [62]. Atherosclerosis is the most common pathological feature of vascular obstruction resulting in thrombotic stroke. Atherosclerotic plaques can undergo pathological changes such as ulcerations, thrombosis, calcifications, and intra-plaque hemorrhage [80, 126]. In addition to atherosclerosis, other pathological conditions that cause thrombotic occlusion of a vessel include clot formation due to hypercoagulable state, fibromuscular dysplasia, arteritis, and dissection of a vessel wall [182].

1.2.2 Embolism

Embolic stroke (ES) can result from embolization of an artery in the central circulation from a variety of sources. Although the heart is the most common source of a thromboembolus, several types of material can be carried to the brain through the cerebral circulation and lodge in a vessel, leading to stroke, including clot, fibrin, pieces of atheromatous plaque, fat, air, tumor or metastasis, bacterial clumps, and foreign bodies.

1.2.3 Systemic Hypoperfusion

A third mechanism of ischemic stroke is systemic hypoperfusion due to a generalized loss of arterial pressure. Several processes can lead to systemic hypoperfusion, the most widely recognized and studied being cardiac arrest due to myocardial infarction and/or arrhythmia. Global ischemia has been shown to cause the largest damage to areas between the major cerebral and cerebellar arteries widely known as the "boundary zone" or "watershed area." The parietal-temporal-occipital triangle located at the junction of the posterior, middle, and anterior cerebral arteries is very frequently affected. Watershed infarction in this area results in a clinical syndrome involving sensory loss in the arm predominantly as well as paralysis; speech and facial muscles are surprisingly spared. These infarcts contribute to about 10 % of total ischemic strokes, and approximately 40 % occur in patients exhibiting carotid stenosis or occlusion.

1.2.4 Cellular Pathophysiology

The brain accounts for 2 % of body weight but 20 % of total oxygen consumption. Approximately 70 % of the metabolic demand in the brain is due to the Na+/K+-ATPase pump that maintains the ion gradient responsible for neuronal membrane potential. Under ischemic conditions, mitochondrial production of ATP ceases and intracellular ATP stores deplete within 2 min. Cell membranes depolarize, leading to a large influx of calcium and sodium and an efflux of potassium. Cells in the infarct core are rapidly and irreversibly destroyed by lipolysis, proteolysis, and disaggregation of microtubules due to metabolic failure. The ischemic penumbra—the zone of tissue between the infarct core and normal brain—experiences diminished blood flow but preserved cellular metabolism. The goal of acute stroke therapies is to normalize perfusion and intervene in the cascade of biochemical dysfunction to preserve the maximal amount of penumbral tissue [42, 46].

At a molecular level, the development of hypoxic–ischemic neuronal injury is greatly influenced by "overreaction" of certain neurotransmitters, primarily

glutamate and aspartate. This process called “excitotoxicity” is triggered by depletion of cellular energy stores. Glutamate, which is normally stored inside the synaptic terminals, is cleared from the extracellular space by an energy-dependent process. The greatly increased concentration of glutamate (and aspartate) in the extracellular space in a depleted energy state results in the opening of calcium channels associated with N-methyl-D-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxanole propionate (AMPA) receptors [175]. Persistent membrane depolarization causes influx of calcium, sodium, and chloride ions and efflux of potassium ions [108]. Intracellular calcium is responsible for activation of a series of destructive enzymes such as proteases, lipases, and endonucleases that allow release of cytokines and other mediators, resulting in the loss of cellular integrity [186]. Inflammatory response to tissue injury is initiated by the rapid production of many different inflammatory mediators, tumor necrosis factor being one of the key agents. Leukocyte recruitment to the ischemic areas occurs as early as 30 min after ischemia and reperfusion [39].

1.2.5 Cell Death After Stroke

Within the center or core of the ischemic territory, blood flow deficits, low ATP levels and energy stores, ionic disruption, and metabolic failure are severe, and cell death progresses in minutes [126]. Infarction is synonymous with necrosis (i.e., cytoplasmic swelling, dissolution of organelles and plasma membranes, and inflammation are present). Necrotic cell death is characterized by energy failure, which results in inhibition of protein synthesis. Therefore, new gene products may not be expressed. In the permanent middle cerebral artery occlusion model in the rat, loss of glucose utilization is rapid and complete within a few hours [184], with little time or energy available for the synthesis of new gene products. Thus, one would surmise that necrosis is the primary mode of cell death in this model. Histologic characteristics of necrotic cell death are cytoplasmic and nuclear swelling, loss of integrity of cell organelles, rupture of the cell membrane, and dissolution of all cell structures. In vivo, necrotic cell death is often accompanied by intense inflammation with recruitment of inflammatory cells. This inflammatory response can injure adjacent normal cells. Although the ischemic core is usually characterized by necrosis, when the ischemia is transient or less severe (penumbra), changes consistent with apoptosis have been described [66]. Apoptosis refers to the morphologic changes that occur after programmed cell death (PCD) [66, 91]. Programmed cell death has several key characteristics: (a) the death process is active, and the expression of new proteins is often involved. (b) Cellular energy stores are normal until the final stages of cellular death; therefore, energy failure is a late, secondary event in programmed cell death. (c) The activation of endonucleases results in numerous double-stranded DNA breaks at the boundaries between histosomes. (d) Morphologic changes characteristic of apoptosis, including cytoplasmic and nuclear budding (“apoptotic bodies”), are present [91]. In contrast to necrosis, programmed cell death results in

neuronal death with little or no accompanying inflammation. Thus, "collateral damage" to neighboring cells is avoided.

1.2.6 Mechanisms of Necrotic Cell Death

There are at least three fundamental mechanisms leading to cell death during ischemic brain injury: excitotoxicity, ionic imbalance, and oxidative/nitrosative stress. These mechanisms demonstrate overlapping and redundant features. They mediate injury within neurons, glia, and vascular elements, and at the subcellular level, they impact the function of mitochondria, nuclei, cell membranes, endoplasmic reticula, and lysosomes [126].

After stroke onset, the loss of energy stores results in ionic imbalance, neurotransmitter release, and inhibition of reuptake (e.g., of glutamate, the major excitatory transmitter in the mammalian brain). Subsequently, binding of glutamate to ionotropic NMDA (N-methyl-D-aspartate) and AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxanole propionate receptor) receptors promotes excessive calcium influx, which triggers an array of downstream phospholipases and proteases that degrade membranes and proteins that are essential for cellular integrity. Mitochondria have also been implicated in toxicity, because of oxygen radical generation and the release of death-inducing factors. In addition, ionotropic glutamate receptors (GluRs) promote an excessive influx of sodium with concomitant cell swelling and edema [126]. Besides calcium, imbalances in other ions are important after ischemia. Large amounts of zinc are stored in vesicles of excitatory neurons and are co-released upon depolarization [21], and loss of zinc from presynaptic terminals correlates with zinc translocation into cell bodies and subsequent neuronal death after focal cerebral ischemia [194]. Recently, imbalances in potassium have also been implicated in ischemic cell death. Neurons express a class of calcium-sensitive high-conductance potassium channels, and compounds that selectively modulate these channels protect the brain against stroke in animal models [67].

The reactive oxygen radical is a key mediator of tissue damage after reperfusion in many organs including the heart, kidney, and brain. Mitochondria are strongly implicated, and this might be due to excessive superoxide production during electron transport and inhibition of mitochondrial electron transport mechanisms by free radicals, leading to even more oxygen radical generation [54]. High calcium, sodium, and ADP levels in ischemic cells stimulate excessive mitochondrial oxygen radical production. Oxygen radicals are also produced during enzymatic conversions, such as the cyclooxygenase-dependent conversion of arachidonic acid to prostanoids and the degradation of hypoxanthine, especially upon reperfusion. Furthermore, free radicals are also generated during the inflammatory response after ischemia. Then, oxidative stress, excitotoxicity, energy failure, and ionic imbalances are inextricably linked and contribute to ischemic cell death. After ischemia and particularly reperfusion, production of reactive oxygen species, including superoxide and hydroxyl radicals, overwhelms endogenous scavenging mechanisms

and directly damages lipids, proteins, nucleic acids, and carbohydrates [54]. Importantly, oxygen radicals and oxidative stress facilitate mitochondrial transition pore (MTP) formation. MTP dissipates the proton motive force that is required for oxidative phosphorylation and ATP generation, and, as a result, mitochondria release their constituents—including apoptosis-related proteins—within the inner and outer mitochondrial membranes [109]. Upon reperfusion and renewed tissue oxygenation, dysfunctional mitochondria might generate oxidative stress and MTP formation [10]. Oxidative and nitrosative stresses are modulated by enzyme systems such as SOD and the NOS family. Mice deficient in expression of the neuronal NOS isoform or the inducible isoform show less tissue damage compared with their wild-type counterparts after cerebral ischemia [84, 86]. Similarly, the generation of nitric oxide and oxidative stress is linked to DNA damage and activation of poly-(ADP-ribose) polymerase (PARP1), a nuclear enzyme that facilitates DNA repair and regulates transcription [38]. In response to DNA strand breaks, PARP1 activity becomes excessive and depletes the cell of NAD+ and possibly ATP. Ischemic cell death by necrotic and apoptotic mechanisms is suppressed by inhibiting PARP1 activity or by deleting the parp1 gene [47].

1.2.7 Mechanisms of Programmed Cell Death

There are at least three sites where PCD can be triggered: the mitochondria, the cell membrane receptors, and the chromosomal DNA [66]. Once PCD is triggered, there are at least three major pathways by which it might be initiated: (1) the intrinsic caspase pathway is activated when cytochrome c is released from the mitochondria and activates caspases; (2) the extrinsic caspase pathway is activated when cell membrane receptor systems, namely, Fas and TNF-α, activate caspases; and (3) the third pathway is activated when AIF initiates apoptosis by caspase-independent mechanisms [66]. Oncogenes Bcl-2 and Bax inhibit and promote PCD, respectively. Caspase-3 acts with Bax and promotes PCD. Treatments aimed at blocking the caspase-dependent and caspase-independent pathways of PCD (novel protease inhibitors) could potentially decrease ischemic brain damage [91].

Just as calcium entry into the neuron is a key step in excitotoxicity, the release of cytochrome c from the mitochondria is a key event in initiating apoptosis in many cell types. Cytosolic cytochrome c complexes with APAF-1 and procaspase-*9* [115]. As a result, procaspase-9 is cleaved into its active form, caspase-9. Caspase-9 then cleaves and activates other caspases, including caspase-3. There is a large body of evidence that brain ischemia can cause activation of caspases. Upregulation and activation of caspase-3 was found to precede death of neurons, especially in the hippocampus and caudate–putamen, in models of transient focal and global brain ischemia [128]. Luo et al. found that deoxyribonuclease activity resulting from transient focal ischemia in the rat could be prevented by inhibitors of caspase-3-like activity [129].

Evidence indicates that many of the mechanisms that initiate programmed cell death are activated in ischemic neurons under certain conditions. The mRNA of the Fas ligand is induced by forebrain ischemia [136]. Expression of the Fas ligand and associated proteins and infarction volumes was smaller in LPR mice that expressed a dysfunctional Fas ligand than in wild-type controls. The Fas receptor is also upregulated after cerebral ischemia in rat brain [52]. TNF-αmRNA transcription is induced as an early response after cerebral ischemia [228]. Expression of the TNF receptor is also increased after cerebral ischemia. TNF-binding protein, a protein that binds and inhibits TNF, reduced infarction volume after middle cerebral artery occlusion in rats [148]. However, ischemic injury was exacerbated in TNF-α -receptor null mice, which suggests that TNF signaling pathways may instead have beneficial effects in ischemic injury under some circumstances. Caspase-8, which is activated by both the Fas and TNF receptors, is expressed and activated after cerebral ischemia [15].

The third pathway by which PCD may occur is caspase-independent. A key factor in this pathway is AIF, a novel proapoptotic molecule that is involved in the final execution of apoptosis and that has been identified and partially characterized [66]. The AIF cDNA codes for a protein of 612 and 613 amino acids in mouse and human, respectively [199]. The AIF protein contains an amino-terminal mitochondrial localization sequence that confines AIF to residing exclusively in the mitochondria in healthy cells [127]. During apoptosis, AIF is released from mitochondria, loses its mitochondrial localization sequence domain (mature AIF), and expresses its apoptogenic effects [199]. When microinjected into the cytoplasm of normal cells, recombinant AIF is sufficient to cause the following four apoptotic hallmarks: (1) the exposure of phosphatidylserine on the plasma membrane surface, (2) the condensation of nuclear chromatin (stage I), (3) large-scale DNA fragmentation, and (4) the dissipation of the mitochondrial transmembrane potential and release of cytochrome c [53]. These apoptogenic effects of ectopic (extramitochondrial) AIF are independent of the action of caspases and are not affected by overexpression of the antiapoptotic protein, Bcl-2 [199].

1.3 Types of Stem Cells Used in Experimental Ischemic Stroke Therapy

Many types of stem cells have been tested and evaluated for their therapeutic potentials in the treatment of ischemic stroke, including mesenchymal stem cells (MSCs), neural stem cells (NSCs), vascular progenitor cells (VPCs), endothelial progenitor cells (EPCs), embryonic stem cells (ESCs), and induced pluripotent stem cell (iPS). The majority of published studies explored the efficacy of transplantation of single type of stem cells [206]. Recently, there are also several studies that investigated the efficacy of transplantation of a combination of different stem cells.

1.3.1 Embryonic Stem Cells (ESCs)

Embryonic stem cells (ESCs) are derived from totipotent cells of the early mammalian embryo [49, 135], which is capable to differentiate not only into all three embryonic germ layers (endoderm, mesoderm, and ectoderm) but also in the embryonic annexes (e.g., placenta, amniotic membranes, etc.), eventually leading to the possibility to obtain any of the more than 220 cell types found in the human organism [68].

The advantage of ESCs is based on its capability of unlimited expansion in vitro to meet the needed amount of cells. In addition, ESCs can be induced to differentiate into neural lineage under specific culturing condition in vitro [8, 153, 172, 243, 258]. Hence, ESCs has been initially considered as an ideal source of transplanted cell for the treatment of neural disorders. Murine ESCs implanted into the contralateral hemisphere following transient cerebral ischemia migrated along the corpus callosum to the ventricular walls, massively populating the border zone of the damaged brain tissue [79], and correlated with improvements in histological and behavioral outcomes [145, 240]. Grafted mouse ESCs also form synaptic connection in the recipient brain [200]. After transplantation of mouse ESCs into rat cortex with a severe focal ischemia, ESC-derived cells expressing cell surface markers of neurons, astrocytes, oligodendrocytes, and endothelial cells could be found in the lesion cavity, and improved structural repair and functional recovery has been demonstrated [230] Intrastriatal transplantation of mouse ESCs or ESC-derived neuron-like cells improved the dopaminergic function and subsequently recovered behavioral dysfunction in focal ischemic rats subjected to middle cerebral artery occlusion (MCAO) [240]. Intracerebral transplantation of mouse ESCs could improve the motor and sensory function of rat with MCAO and reduce the infarct size [200].

Regarding the therapeutic use of hESCs, theoretically they can be differentiated into any type of cell that forms the organism; the undifferentiated ESCs tend to generate teratomas or even highly malignant teratocarcinomas in intact animals [173, 209] and in the stroke rodents [48, 145]. However, xenotransplantation (i.e., stem cells derived from a different species) appears to suppress tumorigenic formation compared to homologous grafting [48] but raises many issues concerning graft rejection. One possible approach is to use in vitro pre-differentiated ESCs that become postmitotic to minimize their tumorigenic potential. Although one group found teratoma formation was independent of pre-differentiation [48], several other groups have found efficacy of pre-differentiated grafts without evidence of tumor formation. ESC-derived neural progenitor cells [59, 75, 202], vascular progenitor cells [160], and mesenchymal stem cells (MSCs) [125] have been shown to exert salutary effects after stroke without noticeable tumorigenesis.

Many studies have explored the effect of ESC-derived neural stem/progenitor cells (NSPC) in animal models of stroke [16, 37, 75, 77, 105]. Most results showed improved behavioral deficit, reduced infarct area, and increased differentiation into neurons after cell transplantation, despite different transplanted cell sources, different stroke animal models, and different infusion routes. However, several stud-

ies found that the grafted human ESC-derived neural cells also have the risk of teratomas formation [14, 192]. Culturing condition might reduce tumorigenesis risk of transplanted ESC-derived neural cells. For example, neural cells derived from human ESCs under defined inductive culturing condition (named SD56) did not show chromosome abnormalities after differentiation and tumor formation after implantation into ischemic rat brains and naive nude rat brains and flanks [37]. Malignant transformation of ESC-derived neural cells has been demonstrated to be related to postischemic environment probably by the stimulation of various local cytokine [181]. It is widely acknowledged that higher cerebral blood vessel density results in less possibility and later occurrence of patients suffering from stroke. Any therapeutic measure aimed at promoting angiogenesis would play a pivotal role in function recovery of stroke patients. Intra-arterial transplantation of human ESC-derived endothelial cells and mural cells significantly increased cerebral blood vessel and vascular density in the ischemic striatum, followed by reduction of the infarct volume and of apoptosis as well as acceleration of neurological recovery in mice with transient MCAO [160].

Currently there are no clinical studies using ESCs for stroke treatment. Several drawbacks of ESCs limit their potential for clinical translation, including ethical concerns, immunological response, limited availability, and heterogeneity of donor cells. These issues need to be resolved to encourage more extensive investigations on ESCs.

1.3.2 Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) are spindle-shaped plastic-adherent cells consisting of a heterogeneous collection of mesenchymal stem and progenitor cells and were first defined as a population of plastic-adherent fibroblastic cells isolated by Percoll density centrifugation [64]. MSCs constitute a population of nonhematopoietic cells in the bone marrow from which these were identified for the first time [58]. MSCs as adult multipotent stem cells can be isolated not only from bone marrow stroma [140] but also from other tissues such as the adipose tissue [185], neural tissue [19], olfactory mucosa [96], heart tissue [138], skin [139], gingiva [56], and many others. Classically, MSCs exhibit CD105, CD73, and CD90 as specific cell surface markers and lack expression of CD45, CD34, CD14 or CD11b, CD79alpha or CD19, and HLA-DR surface molecules. Also, MSCs have the ability to differentiate into adipocytes, osteoblasts, and chondroblasts [45]. Moreover, studies show that they can also differentiate into non-mesenchymal cells such as pancreatic islets [139], neuron-like cells [96], and hepatocytes [197].

Mounting evidence indicated that these MSCs are a favorable cell type for autologous cell transplantations in the scenario of stroke [81]. Though MSCs from different sources are not entirely the same [179], their therapeutic difference for stroke treatment is still unclear. We will briefly review MSCs derived from the bone marrow, adipose tissue, and placenta.

1.3.3 Bone Marrow-Derived MSCs (BMSCs)

In animal models, post-stroke human BMSC transplantation improved sensorimotor function [22, 25, 83, 260], enhanced synaptogenesis, stimulated nerve regeneration [212], decreased tissue plasminogen activator (tPA)-induced brain damage [121], and mediated immunomodulatory effects [245]. Transplanted adult BMSCs migrate to damaged tissue in the brain and decrease post-stroke functional deficits [116, 118]. Migration may be aided by the disruption of the blood brain barrier (BBB) to allow selective entry of BMSCs into the ischemic brain compared to normal cerebral tissue. BMSCs have been reported to stimulate tPA secretion by astrocytes through downregulation of tPA inhibitor plasminogen activator inhibitor-1 [236]. In fact, endogenous tPA could promote BMSC-induced outgrowth of neurites [183] to result in increased synaptic plasticity that correlates with improved functional outcome [236].

In focal ischemic models, BMSC therapy could decrease apoptosis and damage to neurons by inhibiting the downregulation of Bcl-2 and surviving [154]. Survivin-transduced BMSCs saw reduced infarction volume, increased transplanted cell survival, improved functional recovery, and increased protective cytokine expressions such as VEGF and bFGF in a rat model of stroke [122]. Additional evidence of BMSCs protecting against stroke was shown through the i.v. administration of male BMSCs into female rats after MCAO. This type of systemic grafting actually led to decreased apoptosis, increased endogenous neurogenesis, and bFGF expression in the internal border zone; the totality of these processes led to improved neurobehavioral recovery [23]. In a model of chronic stroke, BMSC transplantation improved white matter survival, which may contribute to improved cognitive outcomes [159].

As there are fewer ethical hurdles to overcome when compared to the use of fetal cells, BMSCs may likely be the best alternative for cell therapy at the present moment. Nonetheless, the time required to obtain BMSCs from the individual himself could be a potential limiting factor for the application in acute stroke patients. Yet, BMSC transplantation seems to be an efficacious and practical therapy as seen from clinical studies [9, 114, 198]. The first of many reports originated from Korea. Bang's group infused autologous BMSCs through i.v. into five chronic stroke patients (while 25 received placebo) and analyzed their neurobehavioral deficits after 1 year. Results showed no adverse transplanted BMSC-related effects, and they concluded that i.v. infusion of autologous BMSCs could enhance neurological recovery [9]. They further performed a long-term study of 5 years to evaluate BMSC transplantation safety and efficacy in 52 patients; 16 patients received BMSC treatment. Similarly, they noted no significant side effects following BMSC infusion. In another similar study conducted in Spain, this phase I/II clinical trial transfused autologous bone marrow mononuclear cells via i.a. at 5 and 9 days after stroke in MCA stroke patients and found improved neurobehavioral outcomes as well as feasibility and safety of the transplantation. At the follow-up after 6 months, they reported the absence of stroke recurrence, adverse effects, tumor formation, and deaths except for two individuals with partial seizures that occurred at 3 months [143].

1.3.4 Adipose-Derived Mesenchymal Stem Cells (AD-MSCs)

An abundant number of fully functional MSCs were discovered in the adipose tissue (AD-MSCs) of all adult body. AD-MSCs are easy to obtain without invasive surgery [211], through procedures such as liposuction or abdominoplasty. AD-MSCs can be routinely isolated by collagenase digestion of adipose tissue and cultured [71]. The isolated cells display surface antigens and phenotype similar (but not identical) to BMSCs [63]. There is evidence that AD-MSCs are as effective as BMSCs following permanent middle cerebral artery occlusion (MCAO) in obtaining improved functional recovery in rats [72, 88]. Their lack of expression of MHC-II facilitates their allogeneic administration and possibly allows MSCs from healthy donors to be stored in biobanks for the treatment of stroke patients during the acute phase of the disease [70].

1.3.5 Placenta-Derived Mesenchymal Stem Cells (PD-MSCs)

MSCs are a part of the adult human bone marrow and are seemingly sparse in number, representing less than 0.01 % of the total peripheral blood cells found in the human body. Moreover, as we age, their numbers and function decrease significantly [195, 196]. Nevertheless, MSCs can be readily collected from the chorion, amnion, and villous stroma of the human placenta, independent of the stage of pregnancy [162], which makes it relatively easy to generate a generous number of MSCs in culture.

PD-MSCs have been shown to expand in vitro without showing phenotypic or karyotypic changes. Those derived from allogeneic sources do not require histocompatible tissue matching and therefore are relatively convenient to use vs. bone marrow- or adipose-derived MSCs [168]. Placental cells including PD-MSCs have been found to express MHC class I chain-related proteins A and B that are ligands that can bind to the NK cell receptor NKG2D to downregulate its actions, thereby conferring a type of immune escape strategy [76]. Taken together, PD-MSCs can be considered feasible for application in the clinic. Similar to BMSCs, PD-MSCs possess multilineage differentiation potential with respect to gene expression patterns, morphology, and cell surface antigen expression [61, 87]. Furthermore, PD-MSCs could differentiate into many cell types including neuronal cells [167], e.g., differentiation of PD-MSCs into dopamine neurons was shown to rescue locomotor activity in a hypoxic-ischemia animal model. PD-MSCs have been shown to orient, migrate, and survive in the IBZ when transplanted via intracerebral [180] or i.v. injection [242] after stroke. In fact, PD-MSCs administered through i.v. just 4 h after MCAO demonstrated reduced infarct volume and improved functional outcome [26]. On the other hand, i.v. infusions of PD-MSCs at 8 and 24 h after MCAO proved more efficacious than a single infusion at 24 h [107]. More importantly, compared with fetal-derived MSCs, PD-MSCs showed more robust protective

effects [107]. Additionally, PD-MSCs have proangiogenic effects seen in mice such as increased VEGF expression and generation of new blood vessels in ischemic limbs [149].

The mechanisms of action of MSCs have been explored on two levels: a peripheral level accounting for reduction of inflammation and immunomodulation and a central level expressed by the effects on neurogenesis, astrocytes, oligondendrocytes, axons, and angiogenesis.

Although MSCs have been demonstrated to be capable of differentiating into cells of neural lineage in vitro and express neuronal or glial markers in ischemic brain of animal models [41, 89, 110, 260], the survival number of grafted and differentiated cells was small. Also there is a controversy about the function of neuronal cells derived from MSCs [57, 90, 165, 219, 232, 251]. Hence, cell replacement might not be mainly responsible for the beneficial effect of MSCs on ischemic brain injury in vivo [73]. MSCs are stimulated to secrete various neurotrophic factors including cytokines, chemokines, and extracellular matrix protein by damaged surrounding environment [216]. The paracrine effect hypothesis has been strengthened by recent evidence that stem cells release extracellular vesicles which elicit similar biological activity to the stem cells themselves [20, 111, 235]. These released extracellular lipid vesicles provide a novel means of intercellular communication [60, 171, 215, 250], and among them a particular importance seems to have exosomes. New data show that MSCs release large amounts of exosomes which mediate the communication of MSCs with other cells [33, 113, 223, 234]. Exosomes are complex "living" structures generated by many cell types containing a multitude of cell surface receptors [241], encapsulating proteins, trophic factors, miRNAs, and RNAs [31, 170, 213]. Secretion of trophic factors by MSCs and/or MSC-stimulated resident cerebral cells has been considered to contribute to the beneficial effects mentioned above. MSCs constitutively express BDNF, which was significantly increased when MSCs were transplanted into MCAO model. MSCs overexpressing BDNF showed stronger therapeutic effects than original MSCs alone [110]. Other neurotrophic factors, such as HGF, VEGF, NGF, bFGF, FGF-2, and IGF-1, have been demonstrated to be implicated in endogenous repair mechanisms mediated by MSCs [23, 24, 117, 221, 229, 252]. The trophic factors might play critical roles in neuroprotection, angiogenesis, synaptogenesis, endogenous neurogenesis, and inflammatory and immune response.

1.3.6 Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (iPSCs) are a type of pluripotent stem cell that can be reprogrammed from somatic cells with defined factors. In 2006, Shinya Yamanaka et al. first convert mouse embryonic or adult fibroblasts into pluripotent stem cells by introducing four factors, Oct3/4, Sox2, c-Myc, and Klf4 [204]. In 2007 three groups used second generation method to successful reprogrammed mouse fibroblasts into iPSCs [133, 156, 231]. Unlike the first generation of iPSC, these cells

could produce viable chimeric mice and could contribute to the germ line, which is the "gold standard" for pluripotent stem cells. The researchers used a different marker Nanog, a gene that is functionally important in ESCs, to select for pluripotent cells. By using this different strategy, the researchers were able to create iPSC that were more similar to ESCs than the first generation of iPSC. In 2007, two independent research groups had successfully induced human fibroblasts into pluripotent stem cells using the pivotal genes [203, 246] Recently, researchers can generate iPSC from human renal epithelial cells in urine and peripheral blood cells [147, 262]. These methods of obtaining donor cells are comparatively less invasive and simple. Because some of the reprogramming factors are oncogenes that bring on a potential tumor risk, the clinical application is not for practice [55, 134]. However, Scientist reported that the generation of iPSC is possible without any genetic alteration of the adult cell. These protocols for the establishment and maintenance of iPSC are both safer and more effective [146, 157, 158]. In 2013, the first trial of stem cells produced from a patient's own body has been approved. Researchers in Japan will use autologous hiPSC-derived retinal pigment epithelium [237] cells to attempt to treat wet age-related macular degeneration.

1.3.6.1 Cell Therapy Using iPSCs for Stroke

iPSCs share similar features compared to ESCs in the morphology, growth properties, high reproduction ability, and pluripotency to differentiate into various types of cells [1, 204]. However, comparing with ESCs, iPSCs could be obtained from cells of any part of an adult patient, avoiding both ethical concerns and reducing the need for immunosuppression. The programed iPSCs cells could specifically differentiate into glutamatergic neurons, motor neurons, and GABAergic neurons [17, 35]. These cells hold great promises for the treatment of various neurological diseases [188, 206]. Wang et al. compared the functionality of the transplanted iPSCs and ESCs in a rat model of cerebral ischemia with use of (18)F-FDG PET imaging [222]. It was shown that both of the cells demonstrated comparable effects in neurological functional and metabolic recovery. Direct injection of mouse embryonic fibroblasts generated iPSCs into damaged areas of rat cortex was shown to significantly decrease the infarct size and improved the motor function in rats with MCAO [30]. Intracerebral transplanted human iPSCs were shown to migrate to the ischemic region and differentiate into neuronal cells in rats with MCAO [93]. After 4–16 days of iPSCs grafting, sensorimotor function of rats has been improved significantly. These findings indicated that iPSC therapy in stroke was effective [263]. The neurorestorative effects of iPSC grafts were comparable with those reported using NSCs [249].

Safety is one major issue in the preclinical cell therapy for stroke. Despite the lack of an ethical problem, depending on the methods used, reprogramming of adult cells to obtain iPSCs may pose the high tumorigenicity of grafted iPSCs [106] that clearly needs to be overcome [233]. The tumorigenicity of pluripotent stem cells (including ESCs and iPSCs) has been well documented in vivo [142, 263]. In a side-

by-side comparison between the teratoma composition of human ESCs and iPSCs, human iPSCs develop teratoma more efficiently and faster than human ESC regardless the site of injection [69]. Kawai et al. reported that iPSCs transplanted into ipsilateral striatum and cortex expanded and formed much larger tumors in mice postischemic brain than in sham-operated brain [103]. Klf4, c-Myc, Oct4 and Sox2, the four reprogramming factors exhibit high expression in iPSC-derived tumors, indicating their highly relevant role in tumorigenicity [103, 263]. Subsequently research demonstrated that increased expression of MMP-9 and activated VEGFR2 in the tumors might be involved in promoting the teratoma formation [239]. Inactivation or deletion of the tumor suppressor p53, which is the master regulator of cancer, significantly increases reprogramming efficiency [134]. Thus, there seems to be a trade-off between reprogramming efficiency and tumor generation [263].

Recent studies focused mostly on direct injection of iPSCs pre-differentiated neural cells for stroke therapy [206]. Somatic cells are reprogrammed to iPSC and then differentiated into the cells required for transplantation. A series of studies showed that iPS-derived neural stem cells (NSCs), neural progenitor cells (NPCs), or neuroepithelial-like stem (NES) were safer and more effective than iPSC [21, 92, 166]. Yuan et al. [248] reported that NSCs induced from human iPSCs using retinoic acid and serum-free medium showed stable neural phenotype. After acute transplantation into the ischemic stroke model, these cells survived, migrated into the ischemic penumbra, differentiated into mature neural cells, and showed beneficial effects on functional recovery. Tornero et al. used human iPSC-derived long-term expandable NES cells for stroke therapy [214]. After intracortical implantation into the stroke-damaged striatum and cortex, the cells integrated into stroke-injured host brain and promoted functional recovery. Transplanted iPSC-derived NPC improved sensorimotor functional 14 day after stroke by providing trophic factors, increasing angiogenesis and neurogenesis, and providing new cells for tissue repair [21]. After translation of human iPSC-derived NPC into the postischemic striatum, the graft cells received host tyrosine hydroxylase-positive afferents and contained developing interneurons and homotopic GABAergic medium spiny neurons that, with time, sent axons to the host substantia nigra [166]. Oki et al. [155] reported that human iPSCS-generated NES, which translated into the stroke-damaged mouse and rat striatum or cortex, could survive without forming tumors for at least 4 months. These studies provide evidence that transplantation of human iPSC-derived cells is a safe and efficient approach to promote recovery after stroke [206].

1.3.6.2 Mechanism of iPSC Therapy in Stroke

One major feature of ischemic stroke caused by large vascular occlusion is the significant reduction of cerebral blood flow in the infarct core and surrounding area. The ischemic penumbra has been defined as a hypoperfused area around the ischemic core that is potentially reversible with a timely intervention [120]. Murine NSCs derived from fetal central nervous tissues were reported to release elevated

levels of proangiogenic factors such as vascular endothelial growth factor [74] and nitric oxide and to promote angiogenesis with increased cerebral blood flow in the ischemic boundary region. Therefore, one reasonable hypothesis is that human iPSC-NSCs might be able to restore cerebral blood flow in the ischemic penumbra through angiogenesis [120]. In addition, human iPSC-NSCs might also exert other effects on the penumbra tissues, such as rescue of apoptotic cells, modulation of stroke-induced inflammation, and enhancement of intrinsic repair mechanism. Further studies are urgently needed to understand the possible mechanisms behind the functional recovery and to provide reliable and strong evidence for translating iPSC-based cell therapy into the clinical setting.

1.3.7 Endothelial Progenitor Cells (EPCs)

Endothelial progenitor cells (EPCs) are immature endothelial cells which circulate in peripheral blood [176]. In 1997, Asahara first isolated Flk-1$^+$/CD34$^+$ cells from human peripheral blood and defined as bone marrow (BM)-derived immature cells with the ability to differentiate into mature endothelial cells [7]. They are halfway in their maturation process to become endothelial cells. Therefore, EPCs possess functional and structural characteristics of both stem cells and mature endothelial cells [176]. During their development, EPCs gradually lose stem cell characteristics and progressively gain endothelial cell characteristics [176]. Therefore, EPC are usually defined as cells expressing both stem cell markers and endothelial cell markers [131]. Most of EPCs quiescently lodge in a microenvironment within the BM, termed the stem cell niche [112]. Upon tissue ischemia, EPCs are mobilized from BM to circulation and migrate toward injured blood vessels and ischemic tissue [97]. The mobilized and recruited EPC participate in endothelial repair and contribute to postnatal angiogenesis and repairing function [101].

In 1999, Takahashi et al. first reported that EPCs are mobilized endogenously in response to hindlimb ischemia [205]. In recent years, more and more evidences show that EPCs are present after ischemic stroke [206]. Clinical studies show that acute stroke induces a transient increase of EPCs [98]. It has been found that level of circulating EPCs is independently predictive of prognosis after ischemic stroke [244]. Taguchi et al. reported that EPC levels gradually increased to the highest level by day 7 after stroke onset and remained significantly above the pre-stroke baseline on days 7 and 14, returning to baseline levels by day 30 [201]. In 48 stroke patients, Sobrino et al. [46] demonstrated that an observed increase of EPC cluster numbers 7 and 90 days after a stroke also related to a good functional outcome [191]. The level of circulated EPCs is also shown to be reduced in various stroke risk factors such as hypertension, hypercholesterolemia, diabetes, and atherosclerosis [29, 217, 261]. Another observational study stated that an increase in EPC levels (measured as EPC-CFUs) after acute ischemic stroke correlated with good functional outcome and reduced infarct growth [191]. More evidences demonstrate that

EPCs not only serve as biomarker but also might offer a new therapeutic strategy for ischemic stroke [201, 261].

EPCs are usually reduced in number and dysfunctional in disease conditions, importantly, the level of EPCs in vivo is limited, comprising just less than 0.0001–0.01 % of the peripheral circulating mononuclear cells, therefore, the transfusion of exogenous EPCs could accelerate the repairing processes after stroke [119]. EPC used for transplantation, in these studies were derived from different sources including bone marrow, cord blood and peripheral blood [112, 261]. Zhang et al. first reported that transplantation of EPC improved cerebral neovascularization after focal cerebral ischemia in the adult mouse [259]. Lately, report showed that injection of EPC isolated from human cord blood decrease cell apoptosis, promote angiogenesis and neurogenesis, and improve functional recovery in rat focal cerebral ischemia model [144]. Transarterial administration of EPC derived from bone marrow reduced infarct volume and neurological deficits in acute focal brain ischemia rat [152]. Fan et al. [50] showed that labeled human EPCs were found around microvessels in the cerebral ischemic boundary 24 h after EPC transplantation, and promote neurovascular repair and improves long-term outcome in mice. All these studies indicate that EPCs could serve as a cellular reservoir for the replacement/repair of dysfunctional ECs in stroke and are promising EPC for the treatment of ischemic stroke [261]. In order to enhance the therapeutic effect, EPC modifications such as gene transfection, ischemia preconditioning and pretreatment have been investigated [247, 261]. In a hind limb ischemic model, combination of intravenous infusion of EPCs overexpressing VEGF with local SDF-1 application showed to be more efficient in improving local blood supply than either of them used alone [247].

Currently, several clinical trials (clinicaltrials.gov identifier: NCT01468064, NCT00535197, NCT01289795) are undergoing to evaluate the feasibility and safety of autologous EPC and CD34+ stem cell transplantation for the treatment of patients with ischemic stroke [206, 261].

The safe aspects of EPC transfusion have been explored in recent years. Although many studies have shown that EPCs have a therapeutic effect on ischemic stroke, still, the release of inflammatory factors may compromise the therapeutic efficacy. It was shown that EPC could produce inflammatory factors such as interleukin-6 receptor, interleukin-8, thrombin, monocyte chemotactic protein-1, and recruit monocytes [218, 261]. These factors can effect on the level, mobilization, and survival of EPCs. Several studies put forward that pharmacological or gene modulation of EPCs before and after transplantation may benefit EPCs with regard to both function and survival [261]. In addition, Bone marrow-derived EPCs are a major determinant of nascent tumor neovascularization [150]. The level of cEPCs has been reported higher in patients with lung, hepatocellular, breast, and colorectal cancers [44]. The evidence indicates that EPCs participate in the neovascularization of tumors and that EPC transfusion to patients with tumors should be avoided.

1.3.7.1 Mechanism of EPC Therapy in Stroke

It is well known that a major pathophysiological event of ischemic stroke is vascular endothelial damage that is induced by various high-risk factors such as hypertension, hyperlipidemia, diabetes, and many more. Evidentially, the development of therapeutic approaches to repair damaged ECs has become an attractive topic of research. EPCs have an ability to differentiate into mature ECs and secrete different protective cytokines and growth factors, such as VEGF, FGF-b, and PDGF-bb, to play a significant role in endothelial homeostasis and repair [238]. While on the other side, they may mediate neighboring-injured ECs with normal structure and function extending into injured sites and performing the function of repair.

Neovascularization is necessary for blood vessel reconstruction and collateral circulation establishment, which are important to deliver nutrients and protectants to the injured tissue for repair. Zhang et al. reported that intravenous bone marrow-derived EPC not only increased the angiogenesis but also increased vasculogenesis at the border of the infarct after focal cerebral ischemia in the adult mouse [259]. EPCs are involved in angiogenesis by secreting an array of growth factors and cytokines, such as VEGF, SDF-1, IGF-1, and G-CSF, which can enhance EC proliferation, reduce cell apoptosis, and recruit endogenous progenitor cells [174]. Several studies have proven that endogenous EPCs participate in the neovascularization via C-X-C chemokine receptor type (CXCR) 4/SDF-1 axis after permanent middle cerebral artery occlusion (MCAO) in rats. Bone marrow-derived EPCs have been shown to account for up to 26% of all ECs in neovascularization. In a way, these findings confirm the idea that EPCs promote the repair and regeneration of injured vessels simultaneously, which refers to the combined action of angiogenesis and vasculogenesis after ischemic stroke.

1.3.8 Neural Stem Cells

Neural stem cells (NSCs) are self-renewing, multipotent cells that generate the main phenotype of the nervous system [190]. There are two basic types of stem cell: adult stem cells, which are limited in their ability to differentiate, and embryonic stem cells (ESCs), which are pluripotent. ESCs are not limited to a particular cell fate; rather they have the capability to differentiate into any cell type [32] ESCs are derived from the inner cell mass of the blastocyst with the potential to self-replicate [3].

NSCs are considered adult stem cells because they are limited in their capability to differentiate. NSCs are generated throughout an adult's life via the process of neurogenesis [164]. Since neurons do not divide within the central nervous system (CNS), NSCs can be differentiated to replace lost or injured neurons or in many cases even glial cells [3]. NSCs are differentiated into new neurons within the SVZ of lateral ventricles, a remnant of the embryonic germinal neuroepithelium, as well as the dentate gyrus of the hippocampus [164].

Adult NSCs were first isolated from mouse striatum in the early 1990s. They are capable of forming multipotent neurospheres when cultured in vitro. Neurospheres can produce self-renewing and proliferating specialized cells. These neurospheres can differentiate to form the specified neurons, glial cells, and oligodendrocytes [164]. In previous studies, cultured neurospheres have been transplanted into the brains of immunodeficient neonatal mice and have shown engraftment, proliferation, and neural differentiation [164].

NSCs are stimulated to begin differentiation via exogenous cues from the microenvironment or stem cell niche. This capability of the NSCs to replace lost or damaged neural cells is called neurogenesis [3]. Some neural cells are migrated from the SVZ along the rostral migratory stream which contains a marrow-like structure with ependymal cells and astrocytes when stimulated. The ependymal cells and astrocytes form glial tubes used by migrating neuroblasts. The astrocytes in the tubes provide support for the migrating cells as well as insulation from electrical and chemical signals released from surrounding cells. The astrocytes are the primary precursors for rapid cell amplification. The neuroblasts form tight chains and migrate toward the specified site of cell damage to repair or replace neural cells. One example is a neuroblast migrating toward the olfactory bulb to differentiate into periglomerular or granule neurons which have a radial migration pattern rather than a tangential one [177].

On the other hand, the dentate gyrus neural stem cells produce excitatory granule neurons which are involved in learning and memory. One example of learning and memory is pattern separation, a cognitive process used to distinguish similar inputs [3].

1.3.8.1 The Attempts to Treat Stroke with NSCs

Two different types of insults can cause ischemic damage to the brain. Cardiac arrest or coronary artery occlusion, which leads to abrupt and near-total interruption of cerebral blood flow, causes selective neuronal death to certain vulnerable neuronal populations such as the pyramidal neurons of hippocampal CA1. In contrast, occlusion of a cerebral artery—that is, stroke—gives rise to irreversible damage in a core region and a partially reversible injury in the surrounding penumbra zone. In animals, so-called global ischemia models mimic the effects of cardiac arrest or coronary artery occlusion, whereas focal ischemia models replicate the consequences of stroke. These models are useful to explore various restorative strategies [11].

Can cell transplants reconstruct neural circuits that have been damaged by ischemic insults and thereby lead to functional recovery? In the case of global ischemia, this has been achieved at least to some extent by transplantation of fetal hippocampal tissue into the damaged hippocampal CA1 area in rats [78]. Significant improvement in this model requires homotypic replacement of the degenerated CA1 cells and establishment of reciprocal graft–host connectivity [78]. In animals subjected to focal ischemia, fetal cortical grafts placed in the infarcted cortical area

receive afferent connections from cortex, thalamus, and subcortical nuclei of the host, whereas efferent projections from the graft to the host brain are sparse [193]. In this model, the grafts were able to promote functional recovery only if the rats were housed in an enriched environment, for reasons that are unclear [137].

To avoid the use of human embryonic tissue, other sources of cells have been tested in ischemia models. In rats with focal ischemia, functional improvement was reported after intrastriatal implantation of neurons derived from a human teratocarcinoma cell line [12, 13]. However, although cells expressing neuronal markers are detected within the grafts, there is no evidence that these cells develop into appropriate striatal neurons in this model. Functional recovery was also observed after global ischemia in rats, following implantation of a mouse hippocampal neuroepithelial cell line within the damaged hippocampus [187]. In this case, grafted cells were demonstrated in the CA1 region, and a minority of them were identified either as astrocytes or neurons, mostly with a pyramidal-like morphology.

Human ES cell-derived NSCs, grafted into the ischemic boundary zone in rats subjected to stroke, have been shown to migrate toward the lesion and improve forelimb performance [37]. Electrophysiological recordings showed functional neuronal properties in the grafted cells and synaptic input from host neurons [36], as has been observed for mouse ES cell-derived precursors implanted in stroke-damaged rat brain [16]. Transplanted human fetal NSCs have also given rise to neurons that migrate toward the ischemic lesion in rodents [104], while human NSCs isolated from embryonic striatum and cortex [100] have generated morphologically mature neurons after transplantation into stroke-damaged rat striatum [38].

Taken together, these findings provide evidence that replacement of functional neurons using stem cell grafts is possible in the stroke-damaged brain.

1.3.8.2 Underlying Mechanisms

The subventricular zone (SVZ) of the lateral ventricle and the dentate gyrus of the hippocampus of adult rodent brains contain neural stem cells that produce neuroblasts [5, 43]. Under physiological conditions, neuroblasts in the SVZ travel via the rostral migratory stream to the olfactory bulb where they differentiate into granule and periglomerular neurons throughout adult life [130]. In the SVZ of adult human brains, neural stem cells are present in a band of astrocytes separated from the ependyma [34, 169, 178]. In experimental stroke, focal cerebral ischemia increases neurogenesis in the ipsilateral SVZ, and neuroblasts emigrate from the SVZ to the ischemic boundary regions of the striatum and cortex where they have the phenotypes of mature neurons [6, 94, 161, 208, 210, 254, 257]. Stroke-induced neurogenesis also takes place in the SVZ and ischemic boundary of adult human brains, even in elderly patients aged 60–87 years [95, 132, 141].

Neurogenesis induced by stroke involves proliferation of neural stem and progenitor cells, differentiation of neural progenitor cells, and migration of neuroblasts to the ischemic boundary where neuroblasts mature into resident neurons and inte-

grate into the parenchymal tissue [207]. In adult mice, gene profile analysis of neural progenitor cells from the SVZ that were isolated by laser-capture microdissection has shown that these cells share more than 70 % of all expressed genes with embryonic cortical neural progenitor cells [2]. In murine neural progenitor cells from the SVZ, stroke activates many genes involved in neurogenesis during embryonic development. The most upregulated genes after stroke are those in the transforming growth factor β superfamily, such as bone morphogenetic protein 8, bone morphogenetic protein type I receptors, and growth differentiation factor 2 [124]. After stroke, adult neural progenitor cells seem to recapture embryonic molecular signals, which probably mediate neuroblast migration and stroke-induced proliferation and differentiation of neural progenitor cells.

In vivo analysis of the cytokinetics of neural progenitor cells has suggested that stroke might trigger actively proliferating neural progenitor cells from the SVZ in adult rodents to repeat the cell cycle kinetics of the embryonic form of these cells [255]. During cortical neurogenesis, cell cycle length is associated with progression of neural progenitor cells from proliferation to neurogenic division, and lengthening of the G1 phase of the neuroepithelial cell cycle activates neuronal differentiation [18, 85]. In rats, studies done in vivo that used cumulative and single S-phase labeling with 5-bromo-2′-deoxyuridine (BrdU) [151] showed that dynamic changes in cell cycle kinetics of neural progenitor cells correlated with the proportion of daughter cells that remained within and left the cell cycle over a period of 2–14 days after stroke [256]. Decreasing the length of the G1 phase of the cell cycle at 2–4 days after stroke was associated with an increase in dividing daughter cells that remained within the cell cycle to expand the SVZ progenitor pool rapidly. By contrast, lengthening the G1 phase at 4–14 days after stroke was accompanied by an increased number of daughter cells that left the cell cycle to differentiate into neurons. These data indicate that stroke triggers dynamic changes in the G1 phase of the actively dividing SVZ cell cycle, resulting in early expansion of a neural progenitor pool and subsequent neuronal differentiation, which leads to increased neurogenesis [256]. Neuroblasts in the ischemic boundary have the phenotypes of mature neurons; [6] by use of the patch clamp technique, new neurons in the ischemic boundary were shown to have the electrophysiological characteristics of mature neurons. These findings suggest that neuroblasts mature into resident neurons and integrate into local neuronal circuitry [102]. However, neurogenesis is diminished after stroke and many newly formed neurons die [6].

Cell-based and pharmacological therapies increase neurogenesis in the ischemic brain. These therapies activate the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway in neural progenitor cells [28, 225]. The PI3K-Akt pathway affects several cellular functions such as cell survival, proliferation, differentiation, and migration [102, 220]. Akt regulates proliferation of neural stem cells and neuronal differentiation in embryonic mice [189, 220] and blockage of Akt activation with a selective PI3K inhibitor decreases proliferation of neural progenitor cells [253]. Therefore, the PI3K-Akt signaling pathway seems to be important in the regulation of neurogenesis enhanced by restorative therapies. However, initiation of the PI3K-Akt signaling pathway could differ with individual therapies. Treatment with bone

marrow mesenchymal cells stimulates brain parenchymal cells to secrete an array of neurotrophic factors, including basic fibroblast growth factor and brain-derived neurotrophic factor, which are known to activate Akt [4]. Erythropoietin activates the PI3K-Akt pathway by interaction with its receptor in neural progenitor cells, whereas phosphodiesterase five inhibitors and statins are thought to activate Akt via increased concentrations of cGMP [27, 224]. Mammalian achaete-scute homolog 1 (Mash1) and neurogenin 1 (Neurog1, also known as Ngn1) are pro-neuronal basic helix-loop-helix (bHLH) transcription factors that mediate differentiation of neural progenitor cells into neurons [99, 163]. Akt regulates the assembly and activity of bHLH-coactivator complexes to promote this differentiation [220]. Inhibition of the PI3K-Akt pathway in neural progenitor cells suppresses expression of Mash1 and Ngn1. As a result, neuronal differentiation induced by erythropoietin and statins is prevented [227]. Small interfering RNA in neural progenitor cells also attenuates expression of endogenous Mash1 and Ngn1, which further minimizes the rise in the neuronal population caused by erythropoietin and statins [226, 227]. These findings indicate that the PI3K-Akt signaling pathway activated by these restorative therapies can trigger pro-neuronal bHLH transcription factors in neural progenitor cells, leading to neuronal, but not astrocytic, differentiation [82, 226, 227].

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Chapter 2
Bone Marrow Stem Cells: Source, Characterization, Isolation, Culture, and Identification

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Abstract Stem cell transplantation offers an exciting new therapeutic avenue for stroke, as many studies have demonstrated favorable results in animal models with various cell types. Among them, the bone marrow stem cells (BMSCs) and mesenchymal stem cells (MSCs) in particular, may have enormous therapeutic potential because they can be harvested from the patients themselves without posing ethical or immunological difficulties. More importantly, BMSCs represent an important stem cell population with multipotent functions, including migration and transport functions to sites of local injuries or tissue damage to support appropriate cell and tissue renewal to replace the damaged areas, which are extremely useful for clinical applications, particularly in regenerative medicine. In this chapter, we summarize the source, characterization, isolation, culture, and identification of BMSCs.

Keywords Bone marrow stem cells • Characterization • Isolation • Culture • Identification

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2.1 Introduction

Many studies have demonstrated favorable results in animal models with various cell types [1–4], which consequently have resulted in several early Phase I and II clinical trials with promising outcomes [5–7]. Therefore, the potential therapeutic impact of stem cell transplantation on regeneration of damaged brain tissue opens up numerous possibilities. A variety of cell types, including embryonic stem (ES) cells, neural stem cells (NSCs), inducible pluripotent (iPS) cells, and bone marrow stromal cells (BMSCs), have been studied as sources for use in transplantation into animal models of stroke. Initially, stem cells seemed to work by a "cell replacement" mechanism. NSCs are favored as donor cells for transplantation, since NSCs can differentiate into neural lineages. However, it is now thought that cell therapy may work mostly by providing trophic support to the injured tissue, fostering neurogenesis and angiogenesis [7]. Importantly, BMSCs may have the largest therapeutic potential among them because they can be harvested from the patients themselves without posing ethical or immunological difficulties [8, 9]. In other words, BSMCs are easy to obtain and expand in culture; using the patient's own BMSCs would eliminate the risk of rejection, and BMSCs have the capacity to migrate to the injury site thereby allowing for systemic administration. There is increasing evidence that transplanted BMSCs enhance functional recovery by differentiating into neural cells and/or by producing various kinds of cytokines or growth factors that can rescue the host neurons [10, 11].

2.2 Source

The bone marrow (BM) consists of a heterogeneous population of stem and progenitor cells including hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), two of the most studied BM-derived stem cells. Additionally, endothelial progenitor cells (EPCs) have also been isolated from the BM [12].

2.2.1 Mesenchymal Stem Cells

The terms mesenchymal stem cells (MSCs) and marrow stromal cells are used interchangeably, referring to a population of plastic adherent fibroblastic cells isolated by Percoll density centrifugation [12]. MSCs have the potential to differentiate into mesodermal cell lineages such as adipocytes, chondroblasts, fibroblasts, osteoblasts, and skeletal myoblasts both in vitro and in vivo. Human MSCs (hMSCs) lack telomerase activity and can only undergo about 18 population doublings (PDs) [13].

MSCs are present in a variety of tissues during development but are frequently seen in the bone marrow of adults. They reside in the bone marrow around blood

vessels (as pericytes), in the fat, skin, and muscle [13, 14]. From these readily available sources, MSCs can be isolated, expanded in culture, and stimulated to differentiate into bone, cartilage, muscle, marrow stroma, tendon, fat, and a variety of other connective tissues.

2.2.2 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are defined as self-renewing cells with the capacity to differentiate into any distinct blood-cell lineages [15]. HSCs are mainly produced by the bone marrow in adults. They could also arise from embryonic stem cells. The generation of blood cells from embryonic stem cells (ESCs) and the manipulation of HSCs continue to provide insights into other stem cell systems.

The most successful HSC production method involves the conditional expression of the HoxB4 gene. Previous studies documented that HoxB4 could enhance the potential of hematopoietic cells. The retroviral-mediated overexpression of HoxB4 enhances hematopoietic repopulation through the enhancement of self-renewing activity without inducing leukemia or abnormal differentiation. In addition, the constitutive expression of HoxB4 enhanced the formation of immature mixed hematopoietic colonies [16].

HoxB4 expression transformed ES cell-derived hematopoietic progenitors into HSCs, and, surprisingly, the transient expression of HoxB4 for several weeks was sufficient for the conversion. That is, the overexpression of HoxB4 for several weeks in hematopoietic progenitors seemed to reprogram the differentiation program from hematopoietic progenitors to immature transplantable hematopoietic stem cells. The other possibility is that HoxB4 expanded the ES-induced HSCs, which have not been detected yet because of the low cell numbers [15].

2.2.3 Endothelial Progenitor Cells

Endothelial progenitor cells, initially described by Asahara [17], are immature endothelial cells that circulate in peripheral blood (PB). EPCs mature as endothelial cells and are important components of the vascular system. In their pioneering study, transplanted EPCs, isolated from human umbilical cord blood (UCB), were found in the endothelium of newly formed vessels in ischemic regions, indicating that a discrete cell population within the human blood participates in the formation of new vessels after ischemia [18, 19].

EPCs circulate in the adult blood, so they could be derived from peripheral blood [20]. A population of cells with similar characteristics can also be derived from the human UCB. Several lines of investigations from both animal and human studies indicate that EPCs principally participate in re-endothelialization during the neo-

vascularization of ischemic organs, suggesting that EPC modulation could be directed toward the treatment of cerebrovascular diseases [12].

Progress in the field has been hampered by the lack of agreed parameters to define EPCs or even to distinguish them from adult differentiated endothelial cells. The definition of what constitutes an EPC remains controversial; monocytic-myeloid cells can transiently express cell surface markers typically found on endothelial cells and thus mimic an endothelial phenotype without necessarily meeting other criteria used to define a progenitor cell such as the ability of a single progenitor cell to give rise to a proliferative colony of cells. Many studies have attempted to identify cell surface markers that are unique to EPCs and distinguish them from mature adult endothelial cells as well as from myeloid-monocytic cells, but these attempts have been fairly futile [20].

Indeed, EPCs have been isolated from such seemingly diverse locations such as the bone marrow, circulating blood, heart, skeletal muscle, adipose tissue, spleen, small intestine, and vascular adventitia. However, until recently, the strongest links have unquestionably been documented with the hematopoietic system, where EPCs have been shown to reside within populations enriched with primitive hematopoietic stem cells, more-committed hematopoietic precursors, and even cells within the myeloid pool. When combined with evidence that cells administered at bone marrow transplantation integrate within normal vasculature, atherosclerotic arteries, and tumor neovessels, it is not surprising that an informal consensus has gradually emerged supposing the bone marrow to be the primary source of EPCs in humans [21].

2.3 Characterization and Identification

2.3.1 MSCs

2.3.1.1 Morphology and Cell Cycle Study

MSCs have morphological properties such as small cell bodies with few long and thin cell processes. Their cell bodies have large round nuclei containing prominent nucleoli surrounded by chromatin particles that are dispersed sparingly to give the nuclei a noticeable appearance [22]. Only a small proportion of MSCs are actively undergoing proliferation (~10 %), while the rest of them are at the G0/G1 phase [23]. Although the check points and length of each phase of the cell cycle have not been determined, the high percentage of G0/G1 cells suggests a high competence of MSC to differentiate. Moreover, the G0/G1 MSC population includes a minor and variable subset of resting quiescent cells, as evidenced by RNA and DNA content [24] or by FACS (Fluorescence activated Cell Sorting) analysis of size and granularity [25].

2.3.1.2 Differentiation Capacity

In early studies in vitro [26], MSCs were characterized as a cell population capable of differentiating into osteoblasts [27], adipocytes [28], and chondrocytes [29]. Shortly after, findings were showing the ability of MSCs to differentiate into other tissues of mesodermal origin such as the tendon and ligament [30], cardiomyocytes, and muscle [31]. In parallel, an increasing number of studies reported a wider ecto- and endodermal differentiation potential of MSCs, which includes the skin [26], retinal pigment epithelium [32], lung [33], hepatocytes [34], renal tubular cells [35], pancreatic islets [35], sebaceous duct cells [36], and neural cells [37, 38]. These studies showed that the phenotypic potential of MSCs was wider than anticipated. MSCs indeed have the capacity to differentiate toward all three lineages, i.e., the ecto-, meso-, and endodermal tissues. It has long been known that MSCs are multipotent cells that can give rise to a wide range of cell types upon their differentiation that ends with a terminal cell [39]. Several in vitro studies have been conducted to assess the differentiation potential of MSCs as well as setting up culture conditions, differentiation stimuli, and methods for the identification of each terminal differentiated phenotype. Minguell and coworkers have summarized these information succinctly [40].

2.3.1.3 Function

As mentioned earlier, MSCs possess the ability to secrete bioactive molecules. Bone marrow MSCs produce growth factors and cytokines that contribute to the formation and function of the stromal microenvironment, which produces inductive/regulatory signals not only for MSCs but also for the development of hematopoietic progenitors and other non-mesenchymal stromal cells present in the bone marrow. This trophic effect is important in the maintenance and regulation of the local microenvironment [41]. Nonetheless, the hematopoietic compartment is highly vascularized. Hematopoiesis takes place around the specialized sinusoids that drain into the central vein. Mature cells translocate from the site of their growth and mature through the wall of the sinusoids by active trans-endothelial migration. The sinusoids are lined with specialized endothelial cells and subendothelial pericytes [42] that have very active phagocytosis and are able to produce growth factors (mainly hematopoietic cytokines). In the hematopoietic niche, stromal cells are involved in the maintenance and regulation of the microenvironment. These effects can be direct or indirect: (1) secreted biomolecules activate intracellular signaling pathways, while (2) signaling is initiated in a neighboring cell resulting in the release of bioactive molecules (paracrine signaling). Examples include G-CSF, M-CSF [43], or the RANK ligand [44]. Furthermore, they are also involved in inflammatory processes. Therefore, it seems that MSCs may play specific roles like taking on an immunomodulatory role in several processes such as transplantation tolerance, autoimmunity, tumor evasion, and even fetal maternal tolerance in the case of a pregnancy.

2.3.1.4 Phenotype and Identification

Monoclonal antibodies that recognize MSC antigens [27, 45] have been instrumental in the characterization of BMSCs [46–48]. MSC markers are not unique as they consist of antigenic phenotypes borrowed from endothelial, muscle, epithelial, and mesenchymal cells. In fact, MSCs do not possess typical hematopoietic markers such as CD45, CD14, and CD34 [23, 49]. An extended cytokine expression profile has been described for MSCs. MSCs produce several hematopoietic and non-hematopoietic growth factors, interleukins and chemokines. While many of these cytokines are constitutively produced, others are only expressed after stimulation [43]. In addition, MSCs express several cytokines and growth factor receptors. There are studies that have demonstrated MSCs to be able to produce a vast array of matrix molecules including fibronectin, laminin, collagen, and proteoglycans [50, 51] as well as the expression of several counter-receptors associated with matrix- and cell-to-cell adhesion interactions. Of particular relevance is the strong expression of CD44 [23, 52], a receptor for various ligands like hyaluronan and osteopontin, which plays a central role in the organization of the extracellular matrix in the marrow and bone, respectively [53, 54].

2.3.2 HSCs

2.3.2.1 Multipotency

HSCs are highly capable of multidirectional differentiation; they can give rise to both the myeloid and lymphoid lineages of blood cells [55]. Myeloid cells include monocytes, macrophages, neutrophils, basophils, eosinophils, erythrocytes, dendritic cells, and megakaryocytes or platelets, while lymphoid cells include T cells, B cells, and natural killer cells [56]. HSCs differentiate into more mature hematopoietic progenitor cells (HPCs) that cease to retain self-renewal capabilities but are still able to give rise to colonies that contain functionally active hematopoietic cells. The earliest HPC for the myeloid and lymphoid lineages are the common myeloid progenitor and common lymphoid progenitor, respectively. The HPCs in more differentiated states awaiting to commit to a particular lineage usually grow into mature cells of the myeloid lineage i.e., monocytes, megakaryocytes, granulocytes, and erythroblasts, or the lymphoid lineage e.g., B cells, T cells, and natural killer cells [57]. A study by MacKey showed that to produce a mature circulating blood cell, the original HSC have had undergone about 17–19.5 divisions [58].

2.3.2.2 Self-Renewal

Differentiation is associated with a loss of self-renewing capacity, but HSCs as a population requires self-renewing to maintain the HSC pool. Stem cell self-renewal is thought to occur in the stem cell niche in the bone marrow, and it is reasonable to assume that key signals present in this niche will be important for self-renewal.

New gene-expression analytical tools presently allow researchers to examine developmental changes in telomeres and telomerase activity. The enzyme telomerase is known for extending the regions of DNA at the end of chromosomes known as telomeres. Importantly, it has been found that telomerase activity is critical for cellular proliferation however, as its enzymatic activity decreases with age, it results in shortened telomeres, which is a strong hypothesis for decreased stem cell renewal. To put this in context, telomerase activity in HSCs is correlated with their self-renewing capabilities [59].

As the process of self-renewal is infrequent and quite impossible to induce in cell culture, researchers still do not have a definite clue as to what regulates HSC self-renewal. HSCs that have been injected into an anemic patient were shown to migrate to the bone marrow and actively divide to supply all types of blood cells and give rise to more HSCs with self-renewing capabilities; this is a mystery that remains to be solved with the use of HSC cultures. Two recent studies cultured mouse HSCs and found a number of important factors such as the stem cell factor and thrombopoietin as well as the signaling molecule gp130, which are critical for the renewal of the HSC pool in culture [60, 61].

More clues on how to increase the numbers of stem cells may arise from examining other animals and various developmental stages. During early developmental stages in the fetal liver, HSCs may undergo more active cell division to increase their numbers, but later in life, they divide far less frequently [62]. Culturing HSCs from 10- to 11-day-old mouse embryos gave rise to the possibility of obtaining a 15-fold increase in HSCs within the first 2 or 3 days after removal of the aorta-gonad-mesonephros (AGM) from the embryos [63]. This finding may be viewed as important as the increase could be attributed to the downstream actions of the number of divisions that were directed by the specific embryonic microenvironment. Hence, determining the cellular compositions of adult-derived HSCs and their microenvironments in the AGM region could be critical for HSC expansion and manipulation for clinical purposes.

In another study, mouse HSCs were maintained for 4–7 weeks when they were grown on a clonal line of cells (AFT024) derived from the stroma, the other major cellular constituent of the bone marrow [64]. However, no one knows which specific factors secreted by the stromal cells maintains the stem cells. If it is true that recreating the stromal environment could expand the number of HSCs, then these specific factors, however complicated, could be worth examining [23].

2.3.2.3 Markers and Identification

During embryogenesis, HSCs migrate from one anatomical site to another. This developmental journey begins at the primitive streak/yolk sac and the AGM region and continues through the fetal liver to their final destination in the bone marrow and in rodents also, the spleen [57, 65, 66]. At different stages of development, the human HSCs show diverse surface markers. HSC development has been characterized in minute detail in the mouse, which serves as a model for understanding human hematopoiesis. For obvious ethical reasons, more functional assays are

available for studying murine HSCs than human HSCs. Human HSCs are studied in vivo by using surrogate heterotransplantation models into immunodeficient mice or intrauterine transplants into sheep. It is, however, clear that there are some considerable differences in the expression of surface markers between mouse and human HSCs, which have been summarize in [67].

2.3.3 EPCs

2.3.3.1 Regenerative Potential of EPCs

After thrombotic microangiopathy [68, 69], EPCs seem to participate in the repair of the injured endothelium. It is not known which physiological or pathological factors influence the homing and differentiation of EPCs, and little is known about the signals that direct circulating EPCs to the sites of injured vessels. Recent studies addressing the integration of EPCs into the mature endothelium found that a small fraction of these cells can also transdifferentiate into smooth muscle cells in vitro [70]. This process seems to be dependent on the presence of the transforming growth factor-β1 and cell-to-cell contact. If this were the case, strategically located EPCs within the vascular endothelium could be an emerging standby tool for the regeneration of surrounding endothelial cells (ECs) or smooth muscle cells subsequent to an injury. Furthermore, EPCs may be involved in the regeneration of the ischemic myocardium by modulation of angiogenesis and myogenesis, cardiomyocyte apoptosis, and remodeling in the ischemic cardiac tissue [71–73]. EPCs have also been reported to participate in cerebral neovascularization after ischemic stroke [74]. Thus, EPCs derived from the hematopoietic tissue of the postnatal bone marrow may possess high regenerative potentials and some characteristics of embryonic stem cells. How these cells, if migrating from the bone marrow to the periphery, remain restricted in the circulation and what signals cause their homing to sites of injured endothelium or extravascular tissue, remains an enigma.

2.3.3.2 Phenotype and Identification

In 1997, the isolation of endothelial progenitors for angiogenesis was first described. Cells that were positive for the marker CD34 were obtained from human peripheral blood with the use of magnetic microbeads. By plating these on a fibronectin-coated surface, the cells differentiated and seem to take on endothelial characteristics [17]. Three markers were then discovered some time later characterizing early EPCs i.e., CD133, CD34, and the vascular endothelial growth factor receptor-2 (VEGFR-2) also known as Flk-1 [75, 76]. CD133, originally known as AC133, is a 120-kDa transmembrane polypeptide expressed in early hematopoietic stem and progenitor cells of the human bone marrow as well as peripheral blood and the fetal liver. Cells possessing the markers CD133, CD34, and VEGFR-2 but not vascular endothelial (VE)-cadherin and the von Willebrand factor (vWF) are predominantly seen in the

bone marrow [17]. Mature EPCs in the adult peripheral circulation, however, have been reported to lose CD133 expression but not CD34 and VEGFR-2. In fact, mature ECs are characterized by high levels of VEGFR-2, VE-cadherin, and vWF [77]. Thus, it is highly suggestive that the loss of CD133 expression could indicate the maturation of circulating EPCs, but the point in which EPCs begin to lose this expression is unclear; it may occur either at the time they are transmigrating from the bone marrow into the systemic circulation or later on in the peripheral circulation. Circulating EPCs possess a wide range of markers with varying levels of expressions, typical of the endothelial lineage. Some of the markers include CD31, CD146, VE-cadherin, vWF, and E-selectin [17, 78, 79]. Taken together, we see at least two types of EPCs that are present in the peripheral blood at the same time wherein they possess different sets of markers, thereby aiding in identification.

2.4 Isolation

2.4.1 MSCs

MSCs have been isolated and characterized from many species including the rat, murine, dog, pig, goat, and horse [80]. Classical methods include differentially adhering to tissue culture substrates [81] and density separation [23].

MSCs were first isolated from bone marrow mononuclear cells based on adherence to tissue culture plastic. Guillot's paper described that the resultant cells were allowed to adhere to a standard tissue culture flask for 72 h in MSC medium. Afterwards, they were washed and passaged with TrypLE-Select upon confluence, cultured under humidified conditions in 5 % CO_2, and routinely cryopreserved in 90 % FCS and 10 % dimethyl sulfoxide (DMSO) [82]. Nonetheless, the whole marrow sample adhesion method can result in the nonspecific isolation of mononuclear cells and activation and/or potential loss of target cells. Density separation methods are complicated and toxic substances can easily contaminate the cultures and affect cell viability.

The density gradient centrifugation method is based on BMSC components that are in the 1.05–1.08 density range separate from other cell density separation method. Commonly used reagents are Percoll and Ficoll. It was suggested that the Percoll gradient density separation protocol was the best in terms of MSC yield and self-renewal potential of the MSCs retrieved, while MSCs retrieved with the Ficoll protocol had the lowest self-renewal capacity [83]. Density-gradient centrifugation requires frequent manual interventions and manipulation, which may lead to a higher risk of microbial contamination. Another disadvantage of density fractionation is that it is more difficult to standardize and may be influenced by the operator's skills.

RBC Lysis with Ammonium Chloride It was reported that the highest BMSCs yields were obtained with RBC lysis, compared with density gradient centrifugation [84]. Horn and colleagues [85] compared RBC lysis with Ficoll density fractionation and untreated whole BM adherent cultures, and found that BMSCs can be

efficiently isolated by RBC lysis. This technique is faster and can be standardized more easily for clinical applications of MSCs [86]. In addition, based on the principles of hypotonic lysis and natural sedimentation, a heterogeneous mixture of bone marrow cells can be isolated [80]. Untreated whole BM adherent cultures are best for rabbit BMSC isolation. Pierini et al. isolated MSCs efficiently by the BD Vacutainer® Cell Preparation Tube™ (CPT) peripheral blood. Surprisingly, a fibroblast-colony-forming unit (CFU-F) assay indicated that with CPT, the number of MSC progenitors was 1.8 times higher compared with the Ficoll gradient separation method [87].

2.4.2 HSCs

A major obstacle to studying HSC biology is that the cells are extremely rare and can only be directly assayed using xenograft assays. Only 1 in 106 cells in the human BM is regarded as transplantable HSC. For more than 30 years, researchers have been uncovering novel markers that when used in combination, significantly enhance the purification of HSCs from the murine and human bone marrows. Nearly 20 years now, the separation methods for HSCs include flow cytometry, immunomagnetic beads, and affinity adsorption, among others.

The advent of flow cytometry has proven to be critical in the identification of mature hematopoietic cells due to the heterogeneous and undefined liquidic nature of the hematopoietic organ. A range of CD34 selection techniques have been developed to purify peripheral blood progenitor cells or the bone marrow prior to transplantation [89]. One successful approach that identified cells with hematopoietic stem and progenitor cell (HSPC) characteristics was the use of fluorescently tagged monoclonal antibodies that bind to specific cell surface proteins. Based on the differential binding of the antibodies to the surface of the cell (high, low, negative), researchers selectively isolated cells bearing a specific cell surface immunophenotype FACS and functionally characterized them through in vivo transplant assays. The second approach that was being employed simultaneously was to select BM cells using supravital dyes that were nontoxic to cells. Using a similar approach to immunophenotypic methods, cells were isolated based on the same high, low, and negative staining criteria and then functionally characterized (see Comprehensive Hematopoietic Stem Cell Isolation Methods).

2.4.3 EPCs

Various isolation procedures of EPCs from different sources could be performed using adherence culture or magnetic microbeads. There are some that find that floating cells exhibited therapeutic potential in a mouse myocardial infarction model showing specific local recruitment to the ischemic border zone [90].

Fluorescence-Activated Cell Sorting (FACS) System Isolation of EPCs from BM or peripheral blood is required for this cell-based therapeutic approach. Among a variety of EPC isolation methods, specifically in the case of experimental animal models, a recent mouse-cultured EPC isolation protocol was developed, which is feasible for obtaining a sufficient number of viable cells for both in vitro and in vivo experiments. The protocol for human CD34+ cell (EPC-rich cell population) isolation from peripheral blood can be characterized by FACS [91].

Immunomagnetic Beads A study by Peichev [75] using CD34 magnetic microbeads for isolation showed rather low amounts of CD133/CD34/VEGFR-2 cells (0.002 % of total PMC), corresponding to a number of approximately 70–210 cells per milliliter of blood [88]. However, there appear to be differences between progenitor cells isolated from the bone marrow or from peripheral blood because multipotent progenitor cells from the bone marrow may represent a more undifferentiated cell type with higher plasticity as compared with the peripheral blood-derived cells.

2.5 Culture

2.5.1 MSCs

Many modern culture techniques still utilize a colony-forming unit-fibroblasts (CFU-F) approach wherein the unpurified bone marrow or Ficoll-purified mononuclear cells of the bone marrow are directly plated onto culture plates [92]. In a span of 24–48 h, MSCs could adhere to tissue culture plastic, but not RBCs or hematopoietic progenitors. One report, however, characterized a population of nonadherent MSCs that were not achieved through direct plating [93]. Flow cytometry-based techniques allow the identification of bone marrow cells using unique surface markers such as STRO-1 [94]. STRO-1^+ cells are observed to be more homogeneous and possess greater adherent and proliferative rates. The differences between MSCs and STRO-1^+ cells, however, are unclear [95]. Immunodepletion using MACS have also been reported for negatively selecting MSCs [96].

2.5.2 EPCs

Isolated cells are cultured in medium containing special factors (e.g., VEGF, EGF, and bovine brain extract) to enhance the proliferation of cells with endothelial-like properties. After the initial adhesion, EPCs start to differentiate and lose progenitor characteristics. They form monolayers with endothelial-like appearances in about 3–4 weeks [78, 97]. EPCs can also form capillary tubes given the right conditions [17, 98, 99]. To differentiate adult human marrow-derived progenitor cells toward

the endothelial lineage, one can seed the cells at high density in serum-free or low-serum medium and with the addition of VEGF. To differentiate into other cell types such as osteoblasts, adipocytes, and chondroblasts, one should use a medium containing more than 10% FCS. EPCs show exponential proliferative rates after remaining 30–60 days in culture, contrasting with the early outgrowth of vessel wall-derived endothelial cells that only have limited proliferative capacities.

2.6 Conclusion

Mesenchymal stem cells, hematopoietic stem cells, and endothelial progenitor cells are the major components of bone marrow stem cells. Bone marrow-derived mesenchymal stem cells come from the early development of the mesoderm and ectoderm. They possess small cell bodies with few cell processes that are long and thin, and the vast majority of cells are at the G0/G1 phase of the cell cycle. The ability of MSCs to differentiate into other tissues of mesodermal origin includes the tendon and ligament, cardiomyocytes, and muscle, among others. In addition, they can secrete bioactive molecules. BMSCs produce growth factors and cytokines that contribute to the formation and function of the stromal microenvironment. Furthermore, they are involved in inflammatory processes. The antigenic phenotype of MSC is not unique in that it borrows features of mesenchymal, endothelial, epithelial, and muscle cells. MSCs do not express the typical hematopoietic antigens, CD45, CD34, and CD14. Isolation methods include differentially adhering to tissue culture substrates, density separation, RBC lysis with ammonium chloride, and immunomagnetic beads.

Hematopoietic stem cells come from embryonic stem cells. Its developmental journey begins in the primitive streak/yolk sac and the aorta-gonad-mesonephros (AGM) region and continues through the fetal liver to their final destination in the bone marrow. HSCs are highly capable of multidirectional differentiation and self-renewal. At different stages of development, HSCs show diverse surface markers; thus the use of flow cytometry and supravital dyes can aid in their isolation.

EPCs can be isolated from the human umbilical cord blood. The bone marrow is the primary source of EPCs in humans [21]. EPCs seem to participate in the repair of the injured endothelium. These also possess high regenerative potentials and some characteristics of embryonic stem cells. CD133, CD34, and the vascular endothelial growth factor receptor-2 were discovered in the early EPC. By adherence culture or magnetic microbeads, one can isolate EPCs. After isolation, the cells are cultured in a medium with specific growth factors (e.g., VEGF, bovine brain extract, and epidermal growth factor) to facilitate the growth of endothelial-like cells.

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Chapter 3
Mobilization and Homing of Bone Marrow Stem Cells After Stroke

Weikai Wang, Lefu Chen, Linhui Ruan, Kunlin Jin, and Qichuan Zhuge

Abstract Generally, bone marrow stem cells (BMSCs) reside in the bone marrow, where the microenvironments maintain a dynamic balance between self-renewal and differentiation. However, BMSCs can also be forced into the blood, a process termed mobilization, which is clinically used to harvest large number of cells for transplantation. On the other side, stroke-induced local and systemic pathological responses also lead to the mobilization of BMSCs to peripheral blood and then "homing" to the damaged regions, which is considered as an important regenerative process. In this chapter, we summarize current understanding of the physiological and pathological mechanisms that guide BMSC mobilization and homing to the damaged brain. The underlying cellular and molecular mechanisms, which largely depend on an interplay between chemokines, chemokine receptors, intracellular signaling, adhesion molecules, and proteases, are also discussed. Increasing the number of BMSC mobilization and homing is critical for the promotion of stroke cell-based therapies.

Keywords Bone marrow • Stem cells • Stroke • Characterization • Mobilization • Homing • Trophic factor

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3.1 Introduction

Stroke remains a leading cause of death in the USA and industrial countries. Due to the sudden occlusion of cerebral blood vessels, brain tissues undergo hypoxia, inflammatory cascades, and ultimately cell necrosis, which is associated with neurological dysfunctions. Over the past decade, stem cell therapy has shown great promise in experimental stroke models [1–3]. In the clinical setting, intravascular route transplantation with a minimal invasion is thought to be practical. However, this peripheral route of cell injection requires mobilization of the cells before the cells secrete their products to the site of injury in order to afford brain plasticity and remodeling. Bone marrow (BM) consists of a heterogeneous group of stem and progenitor cells [4], including hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs) and very small embryonic-like stem cells (VSELs). Previous investigations have demonstrated the differentiation of BMSCs into neural lineage cells [5], and their secretion of growth/trophic factors for neuronal survivals [6–8]. This forms the basis of BMSC transplantation or mobilization for therapy in neurological disorders including stroke.

BMSCs mainly resides within BM under multiple regulations of bone marrow niche components, with only a fraction of these cells circulating in the peripheral blood (PB) [9]. Stroke evokes the release of inflammatory cytokines/chemokines and also causes the excitation of sympathetic nervous system to modulate the microenvironments in the bone marrow niches. These mechanisms mobilize and shift BMSCs into the PB. Injured brain also produces factors, which eventually guide the migration of BMSCs to the brain lesions. In this chapter, we will discuss the mechanisms of BMSC mobilization and homing, especially under stroke condition. Introduction of the clinical and experiment applications of reagents to enhance mobilization and homing for better therapeutic effects on stroke would also be highlighted.

3.2 Bone Mesenchymal Stem Cells

3.2.1 The Characterization of BMSCs

BMSCs are multipotent, self-renewing cells that can differentiate into many different types of tissues, such as bones, fat, cartilage, neurons and glial cells. According to the criteria proposed by the International Society for Cellular Therapy (ISCT), BMSCs must express CD105, CD90, and CD73 without the expression of pan-leukocyte (CD45), primitive hematopoietic (CD34), monocytic (CD14 or CD11b), or B-cell (CD79a or CD19) marker. Steady-state BMSCs should also lack the expression of HLA-DR [10].

Among the three positive expressed surface antigens, only CD105 can be found in the uncultured (fresh) human MSPCs [11]. However, those fresh MSCs expressing CD105 are not capable of differentiating into other cells or self-renewing.

It is commonly acknowledged that adhesion protein CD44 is highly expressed in vitro in MSPCs [12, 13]. However, recent study has suggested that, the CD44 fraction contains almost all the primary functional mesenchymal cells in both mice and humans, and these cells acquired CD44 during in vitro culture. This may explain the previous misunderstanding of CD44 expression [14]. Changing of expression of surface markers may coincide with the cell differentiation in vitro culture.

Although, tons of researches have revealed lots of facts concerning characterization of BMSCs, still some detailed and underlying truth about BMSCs remains unknown. Further researches may give us the answer.

3.2.2 BMSC Mobilization After Stroke

The mobilization of MSCs establishes a rich circulating stem cell pool in the peripheral blood, which provides a convenient approach to harvest the cell for autogenic or allogenic transplantation, as well as promotes the cell migration (homing) to injured tissue. Studies have attempted to mobilize MSC into the peripheral blood (PB) using hematopoietic growth factors such as granulocyte-colony stimulating factor (G-CSF). However, most of the investigations brought about disappointing results [15–18], and little evidence showed feasible and efficacious strategies for MSCs mobilization [17–20].

To date, the mobilization of MSC is largely observed in animals undergoing hypoxia. This makes hypoxic condition is an important factor [21, 22]. For example, hypoxic-ischemic brain damaged mice show increased MSCs in the circulation [23]. Although the precise molecular mechanisms underlying hypoxia-induced MSC mobilization are not completely understood, to the best of our knowledge, hypoxia-inducible factor-1 (HIF-1) signaling is an essential mediator [21]. HIF-1 is a transcription factor in response to hypoxia in an organism and mediates homeostatic regulation under low oxygen conditions [24]. HIF-1 is a heterodimer composed of α and β subunits. Under normoxic conditions, the unstable α subunit is prone to hydroxylation, which leads to polyubiquitination and degradation by proteasome; in contrast, hypoxic conditions prevent the α subunits from hydroxylation, maintaining the active function of HIF-1 in the nucleus [25, 26]. Further studies have revealed that HIF-1-induced synthesis of vascular endothelial growth factor (VEGF) might be the direct regulatory molecule for mobilization. VEGF promotes the vasodilation, vascular permeability [27, 28], and angiogenesis [29, 30], which facilitates MSCs drifting from the BM into the PB. However, as VEGF was upregulated during day 2 to day 7 of hypoxia, there is no elevation of VEGF in chronic hypoxic animals, indicating that MSC mobilization in the chronic phase is probably controlled by other factors [21]. In addition to VEGF, stromal cell-derived factor-1 (SDF-1) is also induced by HIF-1 [21, 31]. SDF-1 (or termed CXCL12) is a potent ligand that binds to CXC chemokine receptor 4 (CXCR4) which is expressed on MSCs, and the interaction of SDF-1/CXCR4 plays an important role in MSC apoptosis, migration, and cytokine secretion [32]. The SDF-1α level in the PB was

increased on day 2 of hypoxia, which induced the MSC egress to the circulation [21]. On the basis of the function of serum SDF-1α, this chemokine has been used as a pharmacological agent for MSC mobilization in mice [23]. SDF-1 was demonstrated to increase in the injured brain tissue and serum after stroke [33–38], but little evidence supported the stroke-induced MSC mobilization by this molecule.

3.2.3 The Potential Signaling Pathway/Mechanisms Underlying BMSC Homing

After mobilization of the BMSCs, how to lead these stem cells to the damaged lesion becomes the main issue. Several years of hard work bring us some possible signaling pathways underlying the BMSC homing. Among them, SDF-1 and CXCR-4 come first. Scientists found that the interaction between stromal cell-derived factor-1 (SDF-1) and CXCR-4 may lead to the BMSC homing to the ischemic penumbra.

SDF-1, also called chemokine CXC ligand 12, can be found in all types of cells presented in the CNS. It works as a conjugation spot with CXCR-4, a G-protein coupled receptor, which is expressed on the surface of many different cells including neurons, astrocytes, BMSCs, and some other stem cells [39]. Recent study revealed SDF-1 is highly upregulated in the ischemic region, especially along with the ischemic boundary zone, peaked by 3–7 days and maintained at least 14 days. Meanwhile, CXCR-4 expression is dramatically increased on the surface of BMSCs under the circumstance of hypoxia. On the other hand, deficiency of CXCR-4 significantly decreases the migration of BMSCs toward the ischemic region, indicating that SDF-1/CXCR-4 plays an important role in regulating the homing of BMSCs [40]. Some studies suggested that these two molecules may also regulate the inflammatory response and angiogenesis thereby enhancing the neurorestorative effect after stroke.

3.2.3.1 MCP-1

Monocyte chemoattractant protein-1 (MCP-1), known as a chemoattractant factor, was suggested to contribute to the homing of the BMSCs. Wang et al. evaluated the amount of MCP-1 from the ischemic brain. Result showed the level of MCP-1 increased significantly at 6 h after MCAO and peaked at 48 h with exponential MSCs migration, indicating MCP-1 plays an important role in BMSC homing [41].

3.2.3.2 CD44 and Selectin

Scientists have verified the significant role selecting has played in the process of BMSC binding to endothelial cells. When the endothelial cells were treated with inhibitor of P-selectin, this binding process was suppressed. Meanwhile, the rolling

of BMSC is enhanced with immobilized P-selectin [42]. CD44 has also been proven to induce BMSC migration [43]. Gokhan Yilmaz et al. have verified the function of selectin and CD44 by monitoring the cell recruitment. The MCAO/R mice were treated with antibody against P- or E-selectin. They also used the CD44-deficient mice to test the role CD44 played in cell migration. The results showed attenuated homing was observed in both antagonists treated and CD44-deficient mice, suggesting that cerebral vascular endothelium provides a pro-adhesive phenotype after ischemic stroke to facilitate the migration of BMSCs, which use both P-selectin and E-selectin to home the cells to the infarct zone. CD44 may contribute as a ligand during the BMSC recruitment [44].

The potential signaling molecules and underlying mechanisms concerning homing of BMSCs are complicated and comprehensive. It is not exactly clear whether increasing the target migration of BMSCs is crucial or not in neurological function recovery. Scientists found significant functional improvement with few targeted BMSCs, indicating that cell homing may not be mandatory for stroke treatment [45]. More studies, relating to the migration of BMSCs and poststroke treatment, need to be done.

3.2.4 BMSCs Induced Neurotrophic Factor Secretion and Restoration of Neural Function

BMSC's transplantation can promote sensorimotor function and neural regeneration, decrease tPA-induced brain damage, regulate immune response, and reduce inflammation after ischemic infarction.

Studies have shown that BMSCs can significantly ameliorate the neurological deficits by neurotrophic factor secretion, which drastically decrease the cell apoptosis and promote the proliferation of endogenous cells in the SVZ [46]. These cytokines can also facilitate functional recovery by inducing angiogenesis, regeneration of dendrites and axons as well as rebuilding the synapse.

BMSCs express various cytokines including Ang 1, basic fibroblast growth factor-2 (bFGF2), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and brain-derived neurotrophic factor (BDNF), as well as glial cell line-derived neurotrophic factor (GDNF) [47–51]. These factors have both paracrine and autocrine activities [52], which can regulate cell survival, proliferation, and differentiation.

Intrastriatal infusion of GDNF can promote cell proliferation in SVZ in MCAO rat models. Recruitment of neuroblasts and the improved survival of neurons are also detected [51]. These findings suggest the significant role GDNF played in enhancing neurogenesis, promoting the proliferation and migration of neuroblasts, and inhibiting cell apoptosis.

Wakabayashi K and his group found out that human insulin-like growth factor 1 (IGF-1) was highly increased after transplanting MSC into MCAO rat model.

Therefore, it leads to endogenous expression of vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) [53].

Recently, the novel treatment strategy for ischemic stroke using exosome-mediated miRNAs attracted scientists' attention. Exosomes are nanoparticles released from cells enhancing intercellular communication. miRNAs are noncoding RNAs that can inhibit translation of mRNA. Studies have shown that BMSCs can secrete exosome-enriched miRNAs which facilitate bidirectional material exchanging between BMSCs and injured cells [54], thus contributing to neurorestorative effect by regulating neurogenesis and mediating transplanted cells differentiating to functional neurons [55].

Recent research has revealed that the microRNA-133b level of BMSCs was dramatically increased when treated with ipsilateral tissue extracted from MCAO rats. Additionally, these miRNAs could transfer to neurons and astrocytes via exosomes which resulted in increased miRNAs in these cells, indicating the intercellular communication between BMSCs and parenchymal cells. As a result, miRNAs could promote neurite outgrowth and remodeling by gene expression regulation, thus to facilitate neurological functional recovery after stroke [56, 57]. This novel strategy paved a promising new way for poststroke therapy and future clinical treatment.

3.3 Bone Marrow-Derived Hematopoietic Stem Cells

3.3.1 The Characterization of HSCs and the Niches

3.3.1.1 The Characterization of HSCs

Hematopoietic stem cells (HSCs) are one of the typical subsets of bone marrow (BM) stem cells, possessing the capacity for self-renewal and differentiation into all blood cells of myeloid and lymphoid lineages [58]. HSCs are mainly distributed in adult bone marrow, umbilical cord blood, as well as in peripheral blood in small numbers [59–61]. After harvesting HSCs from these sources, it is an indispensable step to assess the cell before scientific research and clinical applications. The most commonly used approach is detecting markers on cell surface. HSCs are negative for the markers that label lineage commitment, and it is termed as Lin^-. However, the markers may vary between species. For example, human HSCs present $CD34^+$, $CD59^+$, Thy1/$CD90^+$, $CD38^{lo/-}$, C-kit/$CD117^+$, and Lin-, while mouse HSCs display $CD34^+$, $CD59^+$, Thy1/$CD90^+$, $CD38^{lo/-}$, C-kit/$CD117^+$, and Lin^-. Actually, even in humans, there are hematopoietic stem cells presenting $CD34^-/CD38^-$ [62, 63]. One advance in defining human HSCs surface marker is the introduction of CD133, for both $CD34^+$ and $CD34^-$ HSCs share this molecule [64].

3.3.1.2 The HSC Niches

In the adult bone marrow, HSCs exist in contact with osteoblasts and vascular endothelial cells, which are considered as two important microenvironments named osteoblastic niches (or endosteal niches) [65, 66] and vascular niches [67]. Osteoblastic niches, comprised of osteoclasts and other mesenchymal-derived stromal cells, including reticular cells, fibroblasts, and adipocytes, create a hypoxic environment ideal for quiescent and homeostatic HSCs; by contrast, vascular niches, contain sinusoidal endothelial cells, reticular cells, and megakaryocytes, lacking osteoblastic cells, play an essential role in supporting injury-mediated HSCs mobilization [68–72].

The activation of HSCs is regulated by the niches through a combination of adhesion molecules, growth factors (GFs), and chemokines. Some essential molecules expressed by the endosteal niches, such as N-cadherin [73–75], osteopontin (OPN) [76, 77], and angiopoietin-1 (Ang-1) [78], contribute to anchoring HSCs to the niches and maintain the cells in a quiescent state. The interplay of vascular cell adhesion molecule 1 (VCAM-1) expressed by vascular niches and its receptor integrin very late antigen 4 (VLA-4) expressed on HSCs is proposed as a mechanism of homing, lodgment, and mobilization of HSCs [79, 80]. HSCs were found adjacent to the stromal cells with a high-level expression of CXCL12 (also called SDF-1) and sinusoidal endothelial cells. These cells were named CXCL12-abundant reticular (CAR) cells [81]. SDF-1, with its receptor CXCR4 on HSPCs, plays a pivotal role in maintaining the quiescent state of the cells allowing the stem cells to preserve a sustained hematopoietic pool [81–84]. Another important factor is transforming growth factor (TGF)-β which controls proliferation, differentiation, apoptosis [85], and cell cycle entry of HSCs [82, 86]. As a result, the quiescent or motile state of HSCs is switchable by the physiological and pathological changes of these GF- or cytokine-mediated signaling within the BM niches. Another important notion is that except for the dysfunctions of BM per se [87–91], the niches are equally influenced by diseases of other systems [92, 93] via neural and/or humoral regulations on these important molecules. Mounting evidence has shown that the changing of these factors caused by pathological states exert effects on the mobilization of HSCs and homing to affected tissues and organs [36, 94, 95].

3.3.2 HSC Mobilization After Stroke

During homeostasis, HSPCs are continuously released into the peripheral blood (PB) but predominantly reside in the bone marrow in a state of quiescence, owing to the anchoring via adhesion interactions to the specialized niches, which inhibit their motility and proliferation [9]. In response to injury or after administration of pharmacologic agents, hematopoietic stem/progenitor cells (HSPCs) quickly become motile into the blood circulation [96–99]. Researches have shown the mobilization of HPSCs from BM into the PB after the onset of some ischemic diseases,

including acute myocardial infarction [100–102] and ischemic stroke [34, 103–105], and also demonstrated the repair effects of these mobilized cells on the diseases. However, clinical HSPC mobilization has not significantly progressed over the past decades, due to the complicity of the mechanisms by which HSPCs egress the BM [106]. Therefore, it is necessary for neural scientific researchers and clinicians to understand how ischemic stroke mobilizes HSPCs into the BM and the current approaches to enhance this process. The mechanism of HSPCs mobilization holds great promise for further exploration and provision of novel stroke therapies.

3.3.2.1 Mechanisms of HSPC Mobilization After Stroke

Although the definite or complete mechanisms of stroke-induced HSPC mobilization are not fully understood, scientists have so far presented explanations in two major aspects: neuronal (sympathetic) and humoral (chemokines, cytokines, growth factors, and the others) regulations [106]. All the related mechanisms are based on the interactions between the stroke-mediated systemic or local responses and the BM niches. Actually, mounting evidence has presented two major patterns in HSC mobilization: (1) increase the SDF-1 level in the circulation [34] and (2) reduce the SDF-1/CXCR4 interaction in BM (such as inhibiting SDF-1 expression or antagonizing CXCR4) [99, 107–109].

3.3.2.1.1 The SDF-1/CXCR4 Interaction

SDF-1, constitutively produced by BM niches, is a potent chemoattractant for HSCs which express its major receptor CXCR4 [110]. In adult life, the SDF-1/CXCR4 axis serves as a key player for retention, seeding, migration, and mobilization of HSPCs [111]. Evidence has indicated that the balance between adhesion and mobilization of hematopoietic cells depends on SDF-1 concentrations in the PB and the BM niches [112]. On this ground, expression and release of SDF-1 by remote site tissues and organs can probably initiate HSC mobilization. Ischemic stroke in both animal models and patients has shown an increased SDF-1 level in the injured brain tissue or the serum [33–38]. Serum SDF-1 change is positively correlated with infarct volume and severity of stroke in patients [36]. This change, combined with the increased numbers of HSCs in the PB after brain ischemia [103, 104], suggests that the stem cells are mobilized by stroke-induced SDF-1 production. A study conducted by William D. Hill et al. further revealed that SDF-1 is primarily secreted by astrocytes and endothelial cells after stroke [35].

However, the mechanisms of stroke-induced SDF-1 overexpression are still ambiguous, while the relationship between ischemia-induced hypoxia and SDF-1 production is being revealed in the further studies. In homeostatic state, high SDF-1 expression is sustained by the hypoxic condition in the BM niche, which leads to the retention of most HSCs. In a similar way, local hypoxia caused by ischemia may also lead to overexpression of SDF-1. Jean-Jacques Lataillade et al. have demonstrated that SDF-1 expression is regulated by the transcription factor hypoxia-

inducible factor-1 (HIF-1) in endothelial cells under a hypoxic condition [113]. This HIF-1 induced SDF-1 expression was later proven in the ischemic stroke [114]. Actually, timely administration of tissue plasminogen activator (tPA) after acute cerebral ischemia, effectively alleviates the vessel occlusion, but cannot promote CD34+ increase in the PB [104], suggesting that SDF-1 might be induced by hypoxic condition.

3.3.2.1.2 Interplays of the Sympathetic Nervous System with SDF-1/CXCR4 and G-CSF

The BM is highly innervated with nerve fibers which extend into the parenchyma and along the blood vessels. Therefore, sympathetic neurotransmitter catecholamines (CAs) can regulate the BM resident cells, either directly by nervous endings or via circulation transference [9, 115]. Stroke-induced organism stress shows a high activation of the sympathetic nervous system (SNS) [116]. The increased CAs act on adrenergic ß-receptors on osteocytes in the niches, leading to the degradation of transcription factor SP1 which inhibit SDF-1 synthesis. Research has shown that HSC mobilization was reduced by sympathectomy or β-adrenergic receptor blockade [106]. Further evidence has shown that in both wild-type and norepinephrine-deficient mice, the administration of ß-adrenergic agonist could promote mobilization [117].

Granulocyte-colony stimulating factor (G-CSF), which was discovered two decades ago as a hematopoietic growth factor for its function in promoting the survival and growth of HSPCs [118], has been applied as an effective HSC mobilizer in clinical trials for stroke therapy [119–121]. Although the exact mechanisms of G-CSF-induced HSC mobilization are not completed revealed, evidence has shown three potential pathways in general: 1) proteases activation, 2) suppression of adhesion molecules, and 3) attenuation of SDF-1/CXCR4 function [122, 123]. For example, researches have shown that G-CSF interrupts HSC anchoring by suppressing SDF-1 expression and activating the protease CD26 which cleaves the amino-terminal dipeptide of SDF-1, thereby inhibiting the SDF-1/CXCR4 interaction [124–127]. Recent studies have found that the mobilization mechanism by SNS is related with this factor. Peripheral noradrenergic neurons can be activated by G-CSF to induce mobilization of HSCs through depression of osteoblasts and downregulation of SDF-1 expression [117, 128]. Other researches on chimeric NOD/SCID mice have demonstrated that repeated stimulation with G-CSF helps to promote catecholaminergic receptor expression on CD34+ progenitor cells, and these receptors increase the cell motility [129, 130]. In summary, G-CSF-mediated HSC mobilization includes direct act on BM stroma and indirect pathway trough SNS.

3.3.2.2 Agents That Enhance the Mobilization of HSCs for Stroke

In order to enhance the outcome of HSPCs on stroke therapy, pharmacologic agents have been used in preclinical experiments and clinical trials, such as G-CSF, stem cell factor (SCF), and granulocyte-macrophage colony-stimulating factor

(GM-CSF). Clinical mobilization of HSPCs is performed by G-CSF administration with or without other agents, which may develop into one of the bases for noninvasive therapy of autologous HSC for brain ischemia. It is observed that both mature and immature HSCs egress from the BM after several days of G-CSF stimulation [106]. Clinical trials have been widely conducted to mobilize CD34+ HSCS as a stroke therapy [120, 121, 131, 132], which actually improve the PB stem cell level. Its administration at an effective dose has been proven safe and tolerated for most patients [131]. Unfortunately, G-CSF is found to cause bone pain experiences and loss of body weight in patients or animals. In addition, G-CSF may fail in sufficient mobilization of HSCs and requires multiple daily subcutaneous injections for several days [133]. To overcome these drawbacks, a salvage treatment has been proposed, that is, a combination of G-CSF and other mobilizing cytokines (GM-CSF and SCF) [97, 134], that relies on a synergistic effect to enhance mobilization [135].

Recently, numerous types of novel agents have been developed to enhance HSC motility, most of which are also regulators for the BM niche (especially CXCR4 antagonists), not acting on HSCs per se. These experimentally studied agents might hold promise in enhancing human HSCs in clinical stroke treatment. AMD3100 (plerixafor), a reversible CXCR4 antagonist, was shown to rapidly mobilize human and murine HSPCs, and it greatly promoted G-CSF-induced mobilization of HSPCs [129, 136]. This synergic effect is also involved with immune regulations, such as complement cascade [137]. Other CXCR4 antagonists or inhibitors include GRO-β [20], GRO-p [138, 139], AMD3465 [140], T-140 (4F-benzoyl-TN14003) [141], POL6326 [142], and VLA-4 inhibitor BIO5192 [143], which are verified effective agents for HSC mobilization in animal models.

3.3.3 HSC Homing to the Ischemic Brain

HSC homing is a process whereby cells in circulation migrate to target tissues, including their origin the BM and other peripheral tissues. Molecular pathways of the formal process are a mirror image of mobilization [106]. Increasing evidence has demonstrated the capability of HSCs to home the injured tissue/organ after mobilization from the BM to the PB [144]. Herein, we mainly discuss the clinical and experimental observations and underlying mechanisms of HSC homing to the brain after stroke and highlight the applications of these mechanisms to promote homing in stroke therapy.

3.3.3.1 Mechanisms of HSC Homing After Stroke

Proper homing of HSCs to ischemic brain provides a means for the restoration of the injured neural tissues and improved functional outcomes. The main speculated mechanism underlying HSC migration to ischemic brain is considered to be the overexpression and secretion of SDF-1 by ischemic tissues, which develops a

gradient in circulation and guides CXCR4+ HSC recruitment from PB to the injured site [144]. The splicing of the SDF-1 mRNA gives rise to the two isoforms SDF-1α and SDF-1β, which are identical except for four C-terminal amino acids [145]. Murine brain ischemia induced SDF-1β overexpression in penumbra endothelial cells and downregulation in non-injured brain areas, which presented a co-occurrence of infiltration of CXCR4-expressing cells from PB, suggesting that SDF-1β plays a more important role in the homing process [33]. In addition, SDF-1 overexpression was found to persist in ischemic brain tissue for at least 4 months after rat middle cerebral artery occlusion (MCAO) [38], which indicts this chemokine might continually guide HSC migration for a long time. The detailed mechanisms are related to several critical steps in the homing process, generally including (1) interaction with vascular endothelium, (2) trans-endothelial migration, and (3) migration to the brain. The whole process involves the interactions of multiple cells, adhesion molecules, cytokines, chemotactic factors, and extracellular matrix (ECM) degrading proteases, among which, stroke-induced cytokine secretions play an important role in directing HSCs into the brain parenchyma [144]. The first step is the recognition and interaction between HSCs and vascular endothelium. Similar to leukocytes in response to inflammatory signals, HSC rolls along the vessels, which is mainly mediated by E-selectin and P-selectin. E-selectin deficiency has been found to cause decreased homing capacity of endothelial progenitor cells in mice, and this effect is reversible after administrating soluble E-selectin (sE-selectin) [146], because sE-selectin upregulates the adhesion molecules (ICAM-1 and VCAM-1) on endothelial cells. Likewise, the vascularization can be improved by the HSCs adhesion to P-selectin endothelial cells [147]. The attachment is subsequently made firm by integrins, such as the VCAM/VLA-4 [79] and ICAM-l/LFA-1 [148]. Researches have shown that stroke brain released increased SDF-1 to the serum [36], and SDF-1 is a promoter of the adhesion of LFA-1 and VLA-4 to the endothelial ligands [149]. Trans-endothelial migration of HSCs requires basement membrane degradation, which is mediated by the matrix-degrading enzymes, such as matrix metalloproteinases (MMPs). Serum MMP2 and MMP9 level are also significantly increased in acute ischemic stroke patients compared to the healthy individuals [150–153], which indicates it might be a potential mechanism for HSC homing after stroke. However, MMP-2 was shown to suppress the activity and expression of SDF-1 and CXCR4 [154] which are crucial for the migration of HSC to the injured brain.

3.3.3.2 Methods of Enhancing HSC Homing to Ischemic Brain

Mobilized HSCs provide a rich stem cell pool in the circulation for the subsequent recruitment into the ischemic brain. Thereby, any method to augment HSC motility is indirectly amplify its homing capacity, and regular mobilizers, especially hematopoietic factors, are most widely employed agents to enhance motility [133]. A novel approach is on the basis of the chemotactic effect of SDF-1 on CXCR4+ HSCs, that is, to increase the local SDF-1 concentration in the ischemic brain by stereotaxic cerebral injection [155]. Some studies are attempting to prevent the

degradation of SDF-1. For example, a protease-resistant form of SDF-1 substitute, termed S-SDF-1 (S4V), has been used to treat myocardial infarction [156]. However, intracerebral injection may cause iatrogenic damage to the injured brain. Another approach is found in an experiment by Woei-Cherng Shyu et al. that intracerebrally transplanted HSCs to stimulate endogenous HSC migration to the brain after cerebral stroke [157], which might be a potential therapy for clinical application. The underlying mechanism proposed partly related - the increased SDF-1 expression in the brain through the interaction between the implanted cells and the host brain tissue as a contributing factor. Actually, directing autogenous HSCs to the brain is a more ideal therapy for stroke compared to allogenic HSC transplantation (HSCT) systemically or intracerebrally, as it avoids some adverse events, such as the occurrence of graft-versus-host disease (GVHD) [158, 159] and side effects on nontarget organs which the HSC may also migrate into. Another adverse event caused by HSCT was reported that 2 days after HSCT for acute lymphoblastic leukemia, acute cerebral ischemia occurred to the patient, which was likely related to the elevated Factor VIII Level [160]. Therefore, novel efficient and safe methods are highly required to guide the HSC to ischemic brain tissue.

3.3.4 Regulations of HSC on Neural Restoration and Neural Function After Stroke

A sudden arterial occlusion and secondary inflammation in cerebral ischemia lead to the necrosis of neurons and glial cells, which results in functional deficits in movement, sensation, and cognition [161, 162]. Emerging evidence has shown that mobilized or transplanted HSCs migrate to the ischemic brain and improve the neurological function [114, 157, 163]. The underlying therapeutic mechanisms of HSCs include paracrine effects and cell differentiation, which contributes to inflammatory response modulation and cell replacement [157, 164]. In as much as allogenic HSCT could provide stroke patients with large numbers of cells, the number of mobilized autogenic HSCs after stroke may not sufficiently improve the patients' condition. Therefore, it is necessary to apply efficient pharmacological agents to mobilize ample HSCs for stroke, additionally, these hematopoietic growth factors, such as G-SCF and SCF could themselves, present other benefits such as neuroprotection and neuro-regeneration after ischemic stroke [110, 134, 165].

The activities of HSCs, including survival, proliferation, mobilization, differentiation, and migration, are under the regulations of numerous cytokines, growth factors and chemokines secreted in BM niches, other tissues or exogenous administrations [166–172]. Evidence has shown that HSPCs can also secrete the growth/trophic factors, such as GM-CSF, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1) [173], some of which possess the capacity of neural development and restoration for the brain [174–178]. On the ground of the present literature, it is proposed that anti-inflammation/apoptosis is the major protective mechanism in the acute phase of

cerebral ischemia [179–182], which helps to reduce the infarct volume and retain more neural functions. Systemic injection of HSCs into stroke mice model showed significant decrease in neuron apoptosis, infarct volumes, and the infiltration of T cells and macrophages in the ischemic hemisphere [164]. Blood flow supply is highly required for the attenuation of infarction and the reconstruction of neurovasculature. The function of HSC in angiogenesis for ischemic stroke is demonstrated by a research that systemically administrated human CD34+ cells to stroke mice, which created a permissive microenvironment for neuro-regeneration [183]. This angiogenesis is probably induced by angiogenic growth factors, such as VEGF and IGF-1 [173]. However, after the cerebral ischemic rat was treated with PB CD34+ cells, another angiogenic growth factor SDF-1 was significantly upregulated in the brain [157]. Since SDF-1 is not produced by HSCs [173], the result suggested that the angiogenesis is more likely the outcome of interactions between HSCs and brain parenchymal cells, instead of the unilateral regulation through factors secreted by HSCs [157]. Aside from promoting novel microvasculature, CD34+ cell-treated neonatal stroke mice showed an augmented diameter of the cerebral blood vessel in the peri-infarct area, which indicated nitric oxide or other undefined vasodilative factors are responsible for this regulation [184]. Neurogenesis is a pivotal process for the improvement of functional outcome after stroke, and HSCs are reported to improve the trophic milieu for neurogenesis after stroke. For example, HSC-treated ischemic brain presented an upregulation of neurotrophic factors, including SDF-1, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial cell line-derived neurotrophic factor (GDNF), and transforming growth factor beta (TGF-β), which are essential molecules for neurogenesis [157]. Another important restoration mechanism is that the microenvironment provided by ischemic brain directs HSCs to differentiate to neural linage cells, including neurons and glial cells. As a consequence, the tissue defect caused by necrotic brain cells can be supplemented, which is particularly beneficial for chronic stroke with permanent cavities. In vitro, treatment of adult human HSCs with conditioned culture medium induced neuron morphology with extending processes and express specific markers of neurons, astrocytes, and neural stem cell [185, 186], which implicated the neural linage differentiation potential of HSCs under proper microenvironment. Woei-Cherng Shyu et al. intracerebrally implanted $CD34^+$ HSCs to rat ischemic brain, and then followed by immunohistochemistry which showed that the cells differentiated into neurons ($NeuN^+$, $Nestin^+$, $MAP\text{-}2^+$), glial cells ($GFAP^+$), and vascular endothelial cells (vWF^+), which enhanced - neuroplastic effects after stroke with subsequent significantly improved neurological functions [157]. Another investigation conducted by the same team showed that sufficient HSCs can be mobilized by G-CSF and home to rat ischemic brain, which promoted the recovery of neural functions. The mechanism is considered to be partly due to differentiation of endogenous HSCs into neurons, glial cells, and vascular endothelial cells, which are essential components of novel neurovasculature units [163]. This pre-clinic experiment has great promise for a noninvasive autologous HSC therapy for cerebral ischemic patients. However, it is not well established whether the differentiated HSCs play a critical role in neuro-restoration by integrating to the host neural circuits, or because

of increased SDF-1/CXCR4 interaction after G-CSF administration [163] since other pathways by G-CSF [187] may also promote neural restoration.

In conclusion, all current investigations lend support to a notion that HSCs play outstanding regulatory roles in the repair of ischemic injured brains. The cross talk between the therapeutic effects of HSCs and the central nervous system (CNS) further stresses on the possibility of its application for clinical treatment strategies for ischemic stroke or even other neurological diseases. The autologous HSCs mobilized by hematopoietic factors may even have a potential applicability, due to their efficient, feasible, and safe properties.

3.4 Bone Marrow-Derived Endothelial Progenitor Cells

3.4.1 Characteristics of EPCs

Endothelial progenitor cells(EPCs) were first isolated from human peripheral blood in 1997 [188]. EPCs express both endothelial cell markers and stem cell markers [189], and also possess the ability to differentiate into mature endothelial cells which maintain vascular homeostasis and promote vasculogenesis. It is well-known that EPCs can be isolated from three different sources including peripheral blood (PB), bone marrow (BM), and umbilical cord blood (UCB). However, bone marrow-derived progenitor cells are considered as the main source of EPCs, although EPCs remain extremely rare in adult PB [190]. The immunological surface markers used for isolating EPCs include both endothelial cell markers such as CD31, vWF, Tie2, KDR, and VE-cadherin and hematopoietic stem cell marker CD34 or CD133 [191, 192]. However, the identification of EPCs remains controversial, and EPCs that originate from different sources express different cell surface markers. Two methods of functional measurements are used to define the phenotypic feature of EPCs. The first one is performed by counting the number of EPC colonies formed after 7 days of culture. These colonies exhibit many endothelial features including expression of CD31, Tie-2, and VEGFR2 [193]. Another approach is to measure the uptake of Dil-labeled acetylated low-density lipoprotein or binding to specific lectins.

At least two different subsets of EPCs have been identified based on culture characters. Early EPCs appear after short-term culture with spindle-shaped morphology and display peak growth at 2–3 weeks. Late EPCs appear after long-term culture with cobblestone-shaped morphology and display proliferative and tubulogenic potential [194]. Molecular analysis of endothelial progenitor cell (EPC) including genome-wide transcriptional profiling, 2D protein electrophoresis, and electron microscopy showed that early and late EPCs have different gene expression signatures [195]. The same research revealed that early EPCs showed a molecular phenotype linked to monocytes, and late EPCs expressed high level of vascular development and angiogenesis-related signaling pathways (Tie2, eNOS, Ephrins). Several studies also indicated that early EPCs produces large amount of growth

factors, which as a result enhances angiogenesis and neovascularization [196, 197], whereas the late EPCs highly expressed KDR and VE-cadherin which contribute to vascular regeneration [198].

3.4.1.1 EPCs and Angiogenesis

Angiogenesis is defined as the formation of new blood vessels due to proliferation and migration of preexisting endothelial cells [199]. The first isolation of endothelial progenitor cells by Asahara T et al. has opened a new era in angiogenesis research [188]. Bone marrow-derived EPCs can home to neovascularization sites and have the capacity to differentiate into endothelial cells [200]. Accumulating evidence have established that EPCs play a key role in the process of angiogenesis. Early EPCs contribute to angiogenesis by producing a variety of growth factors including IGF-1, G-CSF, VEGF, and SDF-1, which can promote endothelial cell proliferation and recruit endogenous progenitor cells [196]. Another research reported that late EPCs also produce several soluble factors and contribute to angiogenesis [201]. Furthermore, late EPCs also have the ability to differentiate into endothelial cells and finally lead to neovascularization [192]. The contribution of EPCs in angiogenesis has also been established in the recovery processes of animal model of ischemic stroke [202, 203].

3.4.2 EPC Mobilization and Homing After Stroke

Mounting evidence suggests that the level of circulating EPCs correlates with the severity of ischemic stroke. A human study by T. Bogoslovsky et al. revealed that high levels of circulating EPCs were indicative of smaller volumes of acute lesion and less lesion growth. They further indicated that the level of circulating EPCs could serve as a marker of acute phase stroke severity [204]. Moreover, the high level of circulating EPC after acute ischemic stroke is associated with good functional outcome [205]. Another research by Joan MF also found that circulating EPC counts peaked at 7 days after acute stroke, and higher counts were related to better outcome at 3 months after stroke [206].

EPCs can be mobilized from bone marrow into the peripheral circulation and are further able to migrate to injured region. The mechanism underlying this process is still not clear. Howbeit, several chemokines and signaling pathways have been reported to play a role in EPC mobilization and homing. Stromal cell-derived factor-1 (SDF-1) and receptor CXCR4 have been established to play a key role in EPC mobilization and homing. The basal level of SDF-1 in bone marrow and in circulation is comparatively low. In ischemic tissue however, the expression of transcription factor hypoxia-inducible factor-1 (HIF-1) is significantly increased due to reduced oxygen tension. Increased HIF-1 expression will in turn activate chemokine SDF-1 [113]. Finally, EPCs are mobilized from bone marrow to peripheral circulation and

then to ischemic region following SDF-1 chemotaxis. Cell surface receptor CXCR4 is also reported to regulate mobilization and homing of endothelial progenitors in response to chemokine SDF-1 [207]. In a recent study, researchers administrated human EPCs intravenously to adult nude mice after ischemic stroke and found EPCs protect the brain from ischemic injury and lead to improvement in neurobehavioral outcomes. Their results also suggested that hEPC homing is mediated by stromal cell-derived factor-1 (SDF-1)/CXCR4 signaling [208]. Granulocyte-colony stimulating factor (G-CSF) has also been reported to mobilize EPCs in patients with coronary artery disease [209]. Another study by Jean-Pierre found that hematopoietic progenitor cell mobilization after administration of G-CSF is due to the proteolytic cleavage of VCAM-1 by neutrophil proteases in the bone marrow [210].

3.5 Conclusion

Stem cell-based tissue restoration has become the major impetus for regenerative medicine in neurological disease including stroke. Bone marrow-derived MSCs, HSCs, and EPCs have been implicated as promising sources of adult stem cells for CNS regeneration. As is reported in mounting investigations, BMSCs promote neural function recovery via several important mechanisms, of which the ability of MSCs to give rise to neuronal cells is one [211]. However, this trans-differentiation process was questioned since in vitro contamination of cell culture media [212, 213] might alter the morphology of MSCs which influences therapeutic effect. Other underlying mechanisms explaining BMSC therapeutic effects include the secretion of growth factors or cytokines [53, 173, 174, 214], anti-inflammatory/anti-apoptotic modulations [179, 180], and EPC-induced angiogenesis [192, 202, 203]. More recently, a keen interest in repairing the BBB after stroke via EPC transplantation has been taken [215].

Stroke-induced BMSC mobilization to the peripheral blood and migration to the injured brain region potentially provides a self-repair process for the patients, with therapeutic outcome dependent on the number of stem cells aggregating in the brain. Although the mechanisms of these processes are not completely understood, the interaction of SDF-1/CXCR4 [21, 36, 38, 113, 144, 154], the stimulation of G-CSF [17–20, 119–121], and the regulation by sympathetic nervous system [106, 116, 117] after stroke have explicated part of the picture. Based on this fundamental understanding, reagents that augment mobilization and in experimental stroke modeling and clinical translation in patients.

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Chapter 4
Interaction of Bone Marrow Stem Cells with Other Cells

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Abstract Transplantation of bone marrow stem cells (BMSCs) as a regenerative cell replacement therapy for stroke holds great promise. However, the mechanisms underlying functional recovery after stroke remain unclear. Clearly, BMSCs benefit the neurological dysfunction in either direct or indirect methods through the interaction with other cells. In this chapter, we review the direct and indirect interactions of transplanted BMSCs with HSCs, immune cells, neural stem cells (NSCs), neurons, astrocytes, and endothelial cells for better understanding of the mechanisms underlying BMSC-mediated neurological function improvement after stroke.

Keywords Transplantation • Bone marrow stem cells • Immune cells • Interaction

4.1 Introduction

Mesenchymal stem cells and hematopoietic stem cells (HSCs) are two major stem cells coexisting in the mammal bone marrow [13]. Bone marrow stem cells (BMSCs) are generally referred to as bone marrow-derived mesenchymal stem cells. Traditionally, these stem cells are harvested based on Friedenstein's procedure [1–4], which are so-called bone marrow stromal cells, but do not strictly meet the criteria for stem cells as there is a lack of convincing evidence to support the "stemness" of these cells [5] even though they have the propensity to differentiate into mesodermal lineage cells such as osteoblasts, chondrocytes, adipocytes, and muscle cells [135]. For example, cellular heterogeneity exists in the population of these isolated cells, and only a few of them contain colony-forming unit-fibroblastic (CFU-F) activity

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[5]. Therefore, we use the term "bone marrow-derived multipotent mesenchymal stromal cells (BM-MSCs)" recommended by the International Society for Cellular Therapy as an alternative definition in this chapter, while the term "mesenchymal stem cells" will strictly be reserved for cells matching the stated criteria [5].

BM-MSCs are capable of promoting the regeneration of tissues such as bone, cartilage, fat, and muscle [4, 6, 7] and can also differentiate into neural cells such as neuron and astrocytes under both in vitro and in vivo conditions [8–12]. In recent years, the important roles of BM-MSCs have been demonstrated in cytological treatment for nervous system diseases through the expression of different neurotrophic and growth factors after transplantation [13]; these include the glial cell line-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) [15–17]. Furthermore, BM-MSCs protect the neurovascular integrity between the basement membrane and astrocyte end feet, which efficiently ameliorates brain damage and significantly enhances functional recovery in animal models of various neurological disorders, since the neurovascular units (NVUs) that are composed of endothelial cells, astrocytes, and neurons are important for the maintenance of homeostasis in the central nervous system (CNS) [18]. Notably, the ability of BM-MSCs to interact with human leukocyte antigen (HLA)-unrelated immune cells and regulate their effects has important implications in transplantation biology. BM-MSCs possess remarkable immunosuppressive properties. The proliferation and function of the major immune cell populations including T cells, B cells, and natural killer (NK) cells can be inhibited by BM-MSCs. Also, BM-MSCs are able to modulate the activities of dendritic cells (DCs) and promote the generation of regulatory T cells (Tregs) both in vitro and in vivo. However, only a few studies have investigated the interaction of BM-MSCs with specific immune cell subtypes [19–21]. Moreover, transplanted BM-MSCs can benefit CNS injury by regulating the fate of NSCs; multipotent NSCs were originally viewed not only as an endogenous source for cellular replacement following CNS injury but also beneficial therapies for neurological immunomodulation of the damaged environment, which set into motion protective mechanisms that limit the degree of damage and enhance endogenous repair mechanisms following neurological insults [22, 23].

In the following sections, we will discuss the direct and indirect interactions of transplanted BM-MSCs with HSCs, immune cells, NSCs, neurons, astrocytes, and endothelial cells to better understand the mechanisms underlying BM-MSC-mediated neurological function reconstruction and preservation of the poststroke brain.

4.2 Bone Marrow Niche

4.2.1 Definition of Niche

The term "niche" refers to a specific and complex multidimensional position in a particular organ that provides both anatomical and functional depth to the concept of stem cells [24]. The niche for a certain type of stem cell is defined as a

tissue-restricted local microenvironment that directly promotes stem cell maintenance and allows integration of signals from the periphery into appropriate stem cell behaviors [24–27]. The significance of the surrounding microenvironment in regulating and determining stem cell fate including stem cell renewal, expansion, and differentiation was first postulated in 1978 by Schofield based on the observation that the colony-forming unit-fibroblastic cells in the spleen (CFU-S) were less robust than the cells within the bone marrow at reconstituting hematopoiesis [26, 28]. Schofield proposed the concept of the niche as the specialized microenvironment housing HSCs in which the association between HSCs and BM-MSCs was seen to form a regulatory unit that was responsible for effective hematopoiesis [28]. Generally, adult or somatic stem cells will have limited functions when being placed in a dysfunctional or ex situ niche. The specific cues from specific sites guarantee the modulation in stem cell conditions when facing physiological challenges. The balance of the dynamic system based on the interplay between stem cells and their corresponding niche contributes to preventing stem cells from depletion and simultaneously protecting the host from overexuberant stem cell proliferation [24]. The imbalance of the dynamic system may accordingly lead to the occurrence of diseases.

4.2.2 HSC Niche in the Bone Marrow

The niche for HSCs in the bone marrow is widely accepted and experimentally proven. Knowledge of the identity and function of the cellular components (e.g., BM-MSCs, endothelial cells, functionally mature stromal cells) comprising the HSC niche has also been dramatically expanded despite certain controversies. The HSC niche is a complex functional unit. The illustration of the adult bone marrow HSC niche can be found in recently published reviews [26, 29]. Throughout the marrow, HSCs are preferentially located in perivascular regions such as sinusoids and arterioles, where CXC chemokine ligand (CXCL) 12 and stem cell factor (SCF) have been enriched to promote HSC maintenance by distinctly defined perivascular stromal cells and endothelial cells [30–36]. Other cells including macrophages, non-myelinating Schwann cells, sympathetic neuronal cells, and osteolineage cells also likely contribute to the niche. For example, macrophages, as functionally mature descendants of HSCs, can feed back to the niche to influence HSCs proliferation or migration, while primitive osteolineage cells might affect the differentiation of certain lymphoid progenitors. Mature/maturing osteolineage cells could also promote the formation of the HSC niche (e.g., control the size of the niche) even though they do not promote the maintenance of HSCs in a direct manner [26, 29]. Notably, BM-MSCs are known as the key components of the HSC niche.

4.3 BM-MSCs Contribute to the HSCs Niche

Recently, technical advances on high-precision histological imaging, targeted cell tracking, and genetic engineering have provided us more remarkable insights into how BM-MSCs and their progeny affect HSC maintenance and how they participate in regulating hematopoiesis. An explosion of interest in uncovering the nature of the HSC niche and its potential constituent is emerging.

For a better view of the HSC niche, attention should first be drawn to identifying HSCs and BM-MSCs. Exploring specific markers expressed by these two types of cells not only helps reliably identifying and distinguishing these cells from whole cell populations but also contributes to a deeper understanding of where they are located and what functions they are performing correspondingly. In the following sections, we will discuss the different roles of multiple BM-MSCs in the HSC niche on the basis of their respective molecular markers.

4.3.1 Identification of HSCs

HSCs were specifically isolated as Thy-1^{lo}Sca-1^{+}Lineage^{-}c-kit^{+} cells or CD34^{-}Sca-1^{+}Lineage^{-}c-kit^{+} cells in early studies [30, 37–39]. Such complex combinations of 10–12 surface markers limit their use in tissue sections. Identifying HSCs with only several markers holds more practical value. Simple combination of cell surface receptors of the SLAM family such as CD150 (positive in HSCs) and CD48 (negative in HSCs) allowed for high purification of HSCs using a two-color stain. About 21 % of injected CD150^{+}CD48^{-} cells yielded long-term multilineage reconstitution, which was similar to the results obtained with Thy-1^{lo}Sca-1^{+}Lineage^{-}c-kit^{+} cells. More so, an enhanced reliability to identify HSCs could be achieved when further combined with the lack of CD41 expression. CD41 distinguished CD150^{+}CD48^{-} HSCs from CD150^{+}CD48^{-} megakaryocytes; about 45 % of injected bone marrow CD150^{+}CD48^{-}CD41^{-} cells were detected to give long-term multilineage reconstitution. Using this method, CD150^{+}CD48^{-}CD41^{-} HSCs were shown to be located adjacent to the sinusoid vessels in the trabecular region of the bone marrow, thus providing evidence for an existence of a perivascular niche for bone marrow HSCs [30]. In a following study, which was deemed to be more precise, 58 % of the CD150^{+}CD48^{-}CD41^{-} cells were further detected adjacent to sinusoids, and up to 92–95 % of them were within five-cell diameters of a sinusoid [31].

4.3.2 Multiple BM-MSCs in the Perivascular HSC Niche

In consideration of the sinusoid dwelling of major HSCs, there appears to exist some key factors that are secreted by the perivascular cells for influencing HSCs.

By showing a severe reduction of HSCs in the adult bone marrow of CXCR4 (the primary physiological receptor for CXCL12) conditionally deficient mice, Sugiyama et al. highlighted the crucial role of CXCL12-CXCR4 signaling in maintaining the HSC pool [33]. CXCL12, also known as stromal cell-derived factor (SDF)-1 or pre-B-cell growth-stimulating factor (PBSF), is mainly expressed by a small population of CXCL12-abundant reticular (CAR) cells, which are predominantly surrounded by sinusoidal endothelial cells, with some also close to the endosteum [33, 40, 41]. Notably, CAR cells were among the population of multipotent stromal progenitors, with a potential to differentiate into adipocytic and osteoblastic cells both in vitro and in vivo [34]. CAR cells were further suggested to colocalize with the majority of HSCs throughout the bone marrow, playing an essential function in promoting HSC cycling and self-renewal [33, 34, 42]. As highly expressing CXCL12, CAR cells could be selectively eliminated or severely reduced by diphtheria toxin (DT) in CXCL12-DTR-GFP mice, in which a transgene encoding DT receptor-green fluorescent protein (DTR-GFP) fusion protein was knocked into the *Cxcl12* locus. In DT-treated CXCL12-DTR-GFP mice (or CAR cell-depleted mice), the number of HSCs was reduced about 50 % in the bone marrow; the expression of cell-cycle-promoting genes, e.g., those encoding cyclin D1, D2, A2, Cdc2a, and Cdc6, was significantly reduced, while mRNA expression of transcriptional repressor Mad1, known to inhibit HSCs proliferation, was markedly elevated in comparison with untreated wild-type mice or untreated CXCL12-DTR-GFP mice. However, the rapid reduction of HSCs in CAR cell-depleted mice cannot be simply explained by the findings that CAR cells maintain HSCs in a proliferative state. An enhanced differentiating capacity of HSCs caused by the short-term ablation of CAR cells should also be taken into consideration. Moreover, CAR cells were the major producers of SCF in the bone marrow. SCF rescued the expression of cyclin D1, D2, and Cdc6 in HSCs. In summary, CAR cells were suggested as marrow stromal adipo-osteogenic progenitors, which generated large amounts of CXCL12 and SCF to contribute to the maintenance of HSCs in the perivascular niche [34].

In addition to CAR cells, nestin$^+$ MSCs that are exclusively distributed in perivascular regions were also proposed to regulate HSCs maintenance. A landmark study by Méndez–Ferrer and colleagues found that the vast majority of CD150$^+$CD48$^-$Lin$^-$ cells (HSCs) were localized within five-cell diameters from and found to be directly adjacent to nestin$^+$ MSCs. During long-term bone marrow culture, nestin$^+$ MSCs were frequently present in areas where hematopoietic progenitors were enriched. Thus, these spatial and physical evidence confirmed the close relationship between nestin$^+$ MSCs and putative HSCs [35]. Strikingly, expressions of core genes regarding HSC maintenance and attraction such as *Cxcl12*, *Kitl* (encoding SCF), *Angpt1* (encoding angiopoietin-1), *IL7* (interleukin-7), *Vcam1* (encoding vascular cell adhesion molecule-1), and *Spp1* (encoding osteopontin) were extremely high in nestin$^+$ MSCs. These genes, except *Spp1*, could be significantly downregulated during the enforced HSCs mobilization or β3-adrenergic receptor (β3-AR) activation [35, 43]. Furthermore, selective depletion of nestin$^+$ MSCs by inducible expression of DTR in adult *Nes-cre^{ERT2}/iDTR* mice, treated with tamoxifen and DT, led to a severe reduction (50 %) of HSCs in the bone marrow but

a proportional and selective increase in the spleen without any detectable differences in cell-cycle profile or apoptotic rate [35]. All the findings above supported the hypothesis that nestin$^+$ MSCs are functional components of the HSC niche, or could at least regulate the mobilization of HSCs toward extramedullary sites.

What should be noted is that the bone marrow resident nestin$^+$ MSCs could be divided into two distinct types on the basis of their GFP expression levels and cellular morphology i.e., Nes-GFP^{bright} and Nes-GFP^{dim} cells. Interestingly, the more abundant Nes-GFP^{dim} MSCs were reticular in shape and mostly associated with sinusoids, which showed a high similarity to CAR cells [33, 44]. Considering the high expression of CXCL12 in nestin$^+$ MSCs [35], it was likely that Nes-GFP^{dim} MSCs were largely overlapped with CAR cells and may even be a subpopulation of CAR cells. However, nestin$^+$ MSCs contained CFU-F activity, possessed the capacity of multilineage differentiation, and harbored high self-renewal activity both in vitro and in vivo [35]. Therefore, it appears that Nes-GFP^{dim} cells were more primitive than CAR cells.

To gain detailed insights into the source of factors for HSC maintenance, Ding et al. made selective deletions of SCF from different subpopulations of bone marrow cells in *Scf^{gfp}* knock in mice [32]. The resulting data showed that SCF was expressed largely by the stromal cells surrounding the sinusoids throughout the bone marrow and leptin receptor (LepR) expression was highly restricted within these SCF-GFP$^+$ perisinusoidal stromal cells. Conditional deletion of SCF in nestin$^+$ stromal cells, hematopoietic cells, and osteoblasts would not affect the frequency and functions of HSCs, while the selective ablation of SCF in LepR-expressing perivascular cells and endothelial cells would cause a marked reduction of HSCs, which indicated a selective requirement of SCF from perivascular cells and endothelial cells in promoting HSC maintenance [32]. As nestin$^+$ MSCs also produced large amounts of SCF [35], the overlapping condition between nestin$^+$ MSCs and LepR$^+$ cells became the subject of intensive investigations. In actuality, Nes-GFP^{dim} MSCs (about 80 %) largely overlapped with perisinusoidal LepR$^+$ cells, whereas no overlap was found between Nes-GFP^{bright} and LepR$^+$ cells [44]. Therefore, multiple perisinusoidal stromal cells including both LepR$^+$ cells and Nes-GFP^{dim} MSCs as well as partial CAR cells may contribute to maintaining the perisinusoidal HSC niche.

Moreover, Nes-GFP^{bright} cells were found exclusively along arterioles and showed significantly higher expression of genes regarding HSC niches in comparison with Nes-GFP^{dim} cells when subjected to RNA sequencing analysis [44]. In contrast, the classical pericyte marker NG2 appeared to largely label arteriolar Nes-GFP^{bright} stromal cells, but not LepR$^+$ Nes-GFP^{dim} and other stromal cells. The dormant HSCs were preferentially associated with arterioles; depletion of NG2$^+$ Nes-GFP^{bright} cells in tamoxifen-DT-treated *NG2-cre^{ERTM}/iDTR* mice not only obviously reduced the HSC pool but also caused HSCs to move away from arterioles and, likewise, switched them into a non-quiescent state. Thus, a bone marrow arteriolar niche for quiescent HSC maintenance that was potentially related to the rare NG2$^+$ Nes-GFP^{bright} stromal cells was strongly proposed [44].

4.4 Interaction Between Immune Cells and BMSCs

4.4.1 T Cells

BMSC transplantation can mediate immunomodulatory effects and reduce neuroinflammation [45, 46]. Several studies have now shown that MSCs in culture can suppress T-cell proliferation and alter the outcome of the immune cells' responses. MSCs suppress proliferation of allogeneic T cells in a MHC-independent manner [14, 47, 48]. Aggarwal et al. first showed that human MSCs reduced TH1 cell-derived interferon-γ (IFN-γ) and increased the secretion of TH2 cell-derived IL-4 and regulatory T cells (Tregs) [49]. Studies have demonstrated that MSCs suppress allogeneic T-cell proliferation in vitro and in vivo [14, 47, 50]. Inhibition of T-cell proliferation by MSCs appears to be mediated by both cell–cell interaction [14, 51, 52] and MSC-derived molecules that are believed to have immunomodulatory actions on T-cell responses e.g., transforming growth factor (TGF)-β1, IL-10 [53], hepatocyte growth factor (HGF) [47], indoleamine 2,3-dioxygenase (IDO) [54], prostaglandin E2 (PGE2) [49], nitric oxide (NO) [55], matrix metalloproteinases (in particular MMP-2 and MMP-9) [56], and human leukocyte antigen (HLA) class I molecule-G5 (HLA-G5) [57]. Moreover, MSCs inhibit stimulated T-cell proliferation by preventing their entry into the S phase of the cell cycle and by mediating irreversible G0/G1 phase arrest [58]. In a recent study, Ghannam et al. found that under inflammatory conditions, MSCs prevented the differentiation of naive $CD4^+$ T cells into Th17 cells and inhibited the function of Th17 cells in vitro by secreting PGE2 [59]. In addition, BMSCs also suppress T-cell activation and prevent expression and activation of a number of inflammatory factors [14]. It was reported that MSCs induced apoptosis of T cells in vitro by inducing NO production [60, 61]. In contrast to the strong inhibitory effects of MSCs on T-cell proliferation, activity, and apoptosis, there are only relatively minor and reversible effects on T-cell function and toxicity [62]. In addition, MSCs differentiated into various mesenchymal lineages do not appear to alter their interaction with T cells [63]. Moreover, differentiated MSCs had immunologic properties similar to the undifferentiated MSCs [64].

Tregs are a subpopulation of T cells that modulate the immune system, maintain tolerance to self-antigens, and control autoimmune disorders [65, 66]. Studies have shown that high levels of functional $CD4^+Foxp3^+$ Treg cells exist in the bone marrow and play important roles in regulating bone function [67–69]. Depletion of Tregs had no effect on MSCs-mediated inhibition of T-cell proliferation [70]. However, a recent study reported that MSCs could induce kidney allograft tolerance by inducing the generation of Tregs in vivo [71]. Additionally, MSCs have been reported to increase the formation of Tregs that are responsible for the inhibition of allogeneic lymphocyte proliferation [59, 72, 73]. It was demonstrated that Tregs inhibit excessive production of pro-inflammatory cytokines and modulate the invasion and/or activation of lymphocytes and microglia in the ischemic brain to prevent secondary infarct growth [74]. Recent studies indicated that BMSCs increased the production of Treg cells via a mechanism involving not only direct cell contact but

also the secretion of soluble factors such as anti-inflammatory TGF-β, IL-10 [53], PGE2 [75], IDO, inducible NO synthase (iNOS), cyclooxygenase 2 (COX-2) [76], HLA-G5 [57], and the upregulation of developmental endothelial locus-1 (Del-1), an endogenous leukocyte-endothelial adhesion inhibitor [77]. BMSCs can also reprogram conventional T cells into Tregs [78].

Gamma delta T (γδT) cells have pivotal roles in the evolution of brain infarction and accompanying neurological deficits after stroke [79]. Activated γδT cells infiltrated the brain after ischemia, which, in turn, produced the pro-inflammatory cytokine IL-17 within hours. MSCs inhibited γδT-cell expansion from peripheral blood mononuclear cells in both cell-to-cell contact and Transwell systems, and MSCs were lysed by activated γδT cells through a T-cell receptor-dependent mechanism [80].

In in vivo studies, allogeneic bone marrow mesenchymal stem cell (allo-BMSC)-based tissue-engineered bone (TEB) has great potential for bone defect repair. The immunogenicities and biological roles of allo-BMSCs are still controversial. A recent finding indicated that allo-BMSCs can induce a transient immunoreaction, which may temporarily delay the osteogenesis of allo-BMSC/scaffold complex in the early stage of in vivo implantation, whereas the long-term engineered bone formation was not affected [81]. However, BMSCs significantly decrease the numbers of T cells and increase the numbers of Tregs as well as productions of pro- and anti-inflammatory cytokines, which play important roles in improving the neural function and decreasing infarct volume after ischemic stroke [82].

To summarize, through the interactions of MSCs with various immune cells, it appears that MSCs inhibit or limit inflammatory responses and promote anti-inflammatory pathways [49]. Although the cellular mechanisms underlying the immunosuppressive effects of MSCs on T cells have been elucidated previously, the underlying molecular mechanisms remain controversial. It is believed that the mechanisms underlying the suppressive effect of MSCs may differ by species [83]. In addition, the degree of the suppressive effect depends on the concentration of the MSCs; a low MSC/lymphocyte ratio is often accompanied by enhanced MSC proliferation [84].

4.4.2 B Cells

Deng et al. first reported that BMSCs had inhibitory effects on the proliferation, activation, and IgG secretion of B lymphocytes in BXSB mouse models of human systemic lupus erythematosus (SLE), with a slight enhancing effect on CD40 expression and an inhibitory effect on CD40L ectopic expression [85]. Similarly, MSCs have been shown to inhibit the proliferation of B cells activated with anti-CD40L, IL-4, IL-2, pokeweed mitogen, and anti-IgG antibodies [58, 86, 87]. In addition, MSCs also reduce the expression of chemokine receptors and immunoglobulin production by stimulated B cells. For example, MSCs can impair B-cell

functions such as antibody production and secretion of the chemokine receptors (CXCR4, CXCR5, and CCR7), which are responsible for chemotaxis to CXCL12 and CXCL13 [87]. MMP-2 is required for bone marrow stromal cell support of chemotaxis [88]. However, exposure to one chemotherapeutic agent, etoposide (VP-16), can result in the reduced ability of BMSCs to support pro-B-cell chemotaxis [88]. Interestingly, MSCs do not appear to alter surface molecules involved in stimulatory cell cooperation such as HLA-DR, CD40, and the B7 family or inhibit the expressions of TNF-α, IFN-γ, IL-4, and IL-10 [49].

The nature of the mechanism involved in this inhibitory effect of MSCs has not yet been elucidated completely [83]. B-cell proliferation is inhibited by MSCs in a dose-dependent manner in vitro [87]. The inhibitory effect is partly attributable to physical contact and soluble factors, which lead to the blockade of the G0/G1 phases of the cell cycle, similar to what occurs with T cells [49].

4.4.3 NK Cells

MSCs have been shown to suppress NK cytotoxicity [89]. A study indicated that MSCs suppressed NK cell cytotoxicity against HLA class I-positive cells more effectively than HLA class I-negative cells [51]. MSCs impair NK cells to secrete the perforin-/granzyme-containing granules and lyse target cells. Conversely, NK cells are able to lyse MSCs even if IL-2-activated NK cell proliferation is inhibited [90]. However, MSCs did not inhibit the lysis of freshly isolated NK cells, and MSCs were not lysed by allogeneic NK cells [91]. Furthermore, numerous studies have shown that MSCs suppress NK cell proliferation and IFN-γ production driven by IL-2 or IL-15, but only partially inhibit the proliferation of activated NK cells [51, 91–93]. The mechanisms underlying the MSC-mediated immunosuppressive effects are still unclear. Cell–cell contact and soluble factors such as TGF-b1 and PGE2 are believed to play a role in the MSC-mediated suppression of NK cell proliferation [51].

4.4.4 Dendritic Cells

The effects of MSCs on the differentiation, maturation, and function of monocyte-derived DCs have also been reported. MSCs inhibit the differentiation of monocytes into immature DCs by reducing the expression of co-stimulatory molecules and hampering the ability of the former to stimulate naive T-cell proliferation and IL-12 secretion [52, 94]. MSCs strongly inhibited the maturation and functioning of DCs by interfering selectively with the generation of immature DCs via inhibitory mediator of MSCs-derived TNF-alpha, PGE2, but not IL-6 [21, 95]. However, the mechanism underlying the upregulation of PGE2 in monocyte-MSC co-cultures remains unclear. A recent study illustrates that MSCs alter the outcome of the

immune response by inhibiting inflammatory DC1 signaling and promoting anti-inflammatory DC2 signaling by altering the cytokine secretion profile of DC subsets [49]. In addition, it was reported that the cell cycle in DCs was arrested in the G0/G1 phase upon interaction with MSCs [96].

4.5 Interaction of Bone Marrow Stem Cells with Neural Stem Cells

4.5.1 In Vitro

A number of studies have supported the notion that MSCs co-cultured with NSCs can influence the fate of NSCs. MSCs can indeed influence the proliferation and differentiation of NSCs indirectly, which may be mediated in part by notch signaling [97]. Using direct connection of NSCs onto the surface of MSCs, BMSCs could not only induce NSCs to differentiate into neurons but also enhance the survival of neurons as well as decrease the number of astrocytes [98]. In addition, other studies reported that MSCs promoted differentiation of NSCs into oligodendrocytes [99] and inhibited astrocyte differentiation [100, 101]. BMSCs mainly effected NSC differentiation, but not proliferation, which was why the oligodendrocyte proportion increased three times and astrocyte proportion decreased onefold, whereas the neuronal proportion remained unchanged [102].

Notch signaling is important in many aspects of CNS development and is most prominently involved in regulating the proliferation and differentiation of NSCs [103–105]. Wang et al. showed that the expressions of Notch1 and Hes1 increased in NSCs co-cultured with MSCs and decreased after adding the Notch signaling inhibitor, DAPT [97]. Similarly, culturing human MSCs with rat NSCs was found to stimulate differentiation into astrocytes and oligodendrocytes, along with increased expressions of Notch and TGFβ signaling in both cell types, suggesting that the differentiation of rat NSCs in the co-culture was driven through the Notch pathway and by increased secretion of soluble factors such as TGFβ1 by the human BMSCs [106].

MSCs are known to secrete many kinds of trophic factors including brain-derived neurotropic factor (BDNF), VEGF, and nerve growth factor (NGF) [107–111]. Earlier studies initially reported that the exposure of MSCs to certain chemicals caused neuron-like morphological changes in vitro [10, 112, 113], although the morphological change to neuron-like cells is due to cytoskeletal rearrangement rather than genuine acquisition of neuronal fate [114, 115], the neuronal differentiation of NSCs induced by BMSCs, and the neurogenic effect of BM-MSCs could be attributed to several factors such as cell surface molecules and extracellular matrix molecules in the microenvironment provided by BMSCs and soluble molecules secreted by BMSCs [116].

Bai et al. also suggested that cell surface interactions are is the primary mechanism by which MSCs influence the fate of NSCs, indicating that the major effect of MSCs on NSCs is mediated through soluble factors and this effect of differentiation of NSCs differs depending on how long the media is conditioned [99]. Ciliary neurotropic factor (CNTF) has a pro-oligodendrogenic effect on NSCs without affecting cell proliferation or survival [100]. Sygnecka and colleagues concluded that a higher BDNF secretion plays a role in primary MSC-mediated promotion of neuronal regeneration and axonal regrowth [117].

BMSCs can also promote the migration of NSC-differentiated cells. One striking effect of BMSC on neurosphere-derived stem cells is the rapid promotion of neural cell migration [99]. Previous research has shown that chemokine and their receptors are not only expressed in the developing brain but also have a role in orienting cell migration, trophic support, proliferation, and/or differentiation [118]. The chemokine (C-C motif) ligand 2 (CCL2) is a key paracrine factor in the neurogenic effects of BM-MSCs on NSCs in the brain [119].

4.5.2 In Vivo

Similar results have been obtained from in vivo studies. Yoo et al. first verified that transplantation of MSCs improved functional recovery along with increased proliferation of NSCs in the SVZ and survival of newly born neuroblasts [120]. Studies also showed that BMSC transplantation stimulates the differentiation and proliferation of NSCs after stroke and dramatically improves neurological function [112, 121]. In addition, the MSCs may transdifferentiate into neural lineages such as neurons and astrocytes in vivo [122]. Transplantation of BM-MSCs into the SVZ stimulated proliferation and maturation of endogenous progenitors toward the neuronal phenotype, indicating that newborn neurons were derived from endogenous neuronal progenitors, but not from the transplanted cells [119]. These findings are in agreement with previous investigations on the stimulatory effect of BM-MSCs on neurogenesis [109, 116, 120].

The enhanced functional recovery after MSC transplantation may reflect effective mobilization of NSCs to the area of insult. An intriguing recent observation was reported that following implantation into the hippocampi of immunodeficient mice, human MSCs stimulated the proliferation and dorsal migration of endogenous BrdU-labeled NSCs, suggesting that the transplanted BMSCs have an effect on the NSC fate in the SVZ and also the hippocampus [109, 120, 123]. Interestingly, cell surface molecules and extracellular matrix molecules on BMSCs were not involved in the neuronal differentiation of NSCs but soluble factors did instead [98]. However, BMSCs are capable of supporting nerve cells and increasing neurogenesis after cerebral ischemia by secreting neurotropic factors such as BDNF and basic fibroblast growth factor (bFGF) [124]. In addition, the presence of MSCs in areas of injury may enhance the number of NSCs recruited to injury sites and promote repair.

The in vitro studies suggest that the signals from MSCs are soluble, and several factors such as PDGF have been shown to promote NSC migration [125, 126].

Recent data have indicated the important role of chemokines such as MCP-1, SDF1, and its receptor CXCR4-CCL2 in migration and differentiation of neural progenitor cells after stroke [127, 128]. For instance, the chemotactic molecule SDF-1 may partially mediate the homing of transplanted BMSCs to the injury sites in the brain and then exert the beneficial effects evoked by BMSC treatment, which may be related to the induction of neurogenesis and reduction of glial scar formation [129].

MSCs may exert their effects on NSCs by attenuating inflammation. A recent study showed that human MSCs have a neuroprotective effect on neurons through anti-inflammatory actions mediated by the modulation of microglial activation, which could have major therapeutic implications in the treatment of neural diseases [130]. In addition, BMSC treatment reduced scar thickness and increased the number of proliferating cells and oligodendrocyte precursor cells of the ipsilateral SVZ [129]. Interestingly, it was revealed that the combination cell therapy of BMSCs and NSCs is more efficient in promoting functional recovery after cerebral stroke, suggesting that these stem cell types could work in synergy to produce additional benefits that would otherwise be absent if they acted alone [131].

4.6 Interrelation of Neuronal Cells and Transplanted BMSCs

BMSCs constitute a heterogeneous collection of mesenchymal stem and progenitor cells. Human BMSCs can transdifferentiate into neural and mesodermal cell lines [132–134]. BMSCs have been found to significantly promote neurite extension of neurons in an organotypic brain slice [135, 136]. Regeneration of the facial nerve was improved by both uBMSC and dBMSC in rats [137]. BMSCs also promoted neurite outgrowth in spinal neurons by secreting soluble factors such as BDNF and GDNF [138].

BMSCs significantly enhance neurogenesis in the SVZ. In fact, some transplanted BMSCs also express neuronal phenotypes in the neocortex, SVZ, corpus callosum, and peri-lesion area [139]. Other studies demonstrated that the transplanted BMSCs can express GABAA receptors and MAP2 in the peri-infarct neocortex, suggesting that the transplanted BMSCs may contribute to migration toward the peri-infarct area and acquire neuron-specific receptor function [140] along with improvement in sensorimotor function [113, 139, 141–144], synaptogenesis and nerve regeneration [145].

Other beneficial effects observed upon BMSC transplantation include enhanced structural neuroplasticity and increased axonal outgrowth from healthy brain tissue

[146]. At 28 days after intracarotid BMSC transplantation, axons, myelin, and white matter bundles were significantly increased in the striatum and corpus callosum. The remodeling of white matter in the cortical IBZ and corpus callosum is increased by axonal sprouting and remyelination [147]. It was revealed that axonal sprouting and remyelination in the cortical penumbra were significantly increased after transplantation of BMSCs directly into the striatum of MCAO mice [148]. Through ^{123}I-iomazenil single photon emission computed tomography, the neuronal integrity in the peri-infarct area can be visualized after the transplantation of BMSCs [149].

BMSC transplantation has the potential to repair the ischemia-damaged neural networks, restore lost neuronal connections [150], and significantly ameliorate the breakdown of neurovascular integrity [18]. Administration of BMSCs significantly increases the axonal restructuring on the deafferented red nucleus and the denervated spinal motor neurons, which increases axonal sprouting and rewiring of the corticospinal tract emanating from the uninjured motor cortex onto denervated spinal cord and the axonal connections from the intact motor cortex to the denervated spinal cord at both the cervical and lumbar levels [151]. Recently, many have reported that BMSCs markedly enhanced interhemispheric, intracortical, thalamocortical circuit connections [150]. More so, cortical neurons surviving in the peri-infarct motor cortex underwent axonal sprouting to restore connections between different cerebral areas [152]. Further, in a rat model of intracerebral hemorrhage, administration of BMSCs can significantly increase neuronal plasticity of the denervated corticospinal tract at bilateral forelimb areas of the cortex [153].

4.7 Interrelation Between Endothelial Cells and Transplanted BMSCs

Under indirect co-culture conditions, endothelial progenitor cells (EPCs) could enhance the proliferation of BMSCs but could not regulate cell apoptosis in vitro [154]. Transplanted BMSCs can selectively migrate to the site of the infarct area, stimulate angiogenesis and arteriogenesis as well as induce a neovascular response that results in a significant increase in local cerebral blood flow to the ischemic area, aid for the repair of the injured brain [155], and reduction of infarct size and blood–brain barrier disruption [156]. Interestingly, collection of transplanted BMSCs from the stroke rat saw significant increases in phosphorylated-Tie2 activity in brain-derived endothelial cells and capillary tube formation compared with BMSCs collected from normal rats [157]. Further, a clinical study showed that in ischemic stroke patients, the number of new vessels surrounding injured tissue correlated with longer survival [158]. Studies have shown the therapeutic potential of angiogenesis in the restoration of local blood flow and functional recovery in ischemic diseases [159].

Recent studies have claimed that appropriate application of pharmaceuticals (such as Z-VAD, SDF-1α, and statins) decreases the apoptosis of grafted BMSCs, induces migration into the ischemic area, modulates the expressions of protein/cell and trophic factors, promotes angiogenesis in the ischemic area, and enhances the therapeutic effect of BMSC transplantation. The intracerebral coadministration of SDF-1α and BMSCs promotes BMSC migration to the ischemic lesion, increases the density of blood vessels in the ischemic cortex, and enhances neuronal plasticity [160]. A further investigation revealed that DETA-NONOate increases the expressions of CXCR4 and MMP in BMSCs and promotes BMSC adhesion and migration to mouse brain endothelial cells and astrocytes [161]. In addition, combined intravenous injection of BMSCs with DETA upregulates angiopoietin-1 and its receptor Tie2 and restores neovascularization, which is regarded as the main therapeutic goal in ischemic stroke. Along with the angiogenesis in the infarct area and the improvement in the microenvironment, the proliferation of neurons and neuroglial cells and the growth of neurofibrils was also observed. These elements are reciprocal factors for the repair of the infarct area [162].

4.8 Interrelation of Glial Cells and Transplanted BMSC

BMSC treatment produced dramatic changes in the number and activation of brain astroglia and microglia, particularly in the region of the infarct [163]. An in vitro study found that BMSC-conditioned medium (BMSC-CM) significantly inhibited proliferation and secretion of pro-inflammatory factors by activated microglia and significantly induced apoptosis of microglia [164]. The intravenous injection of BMSCs noticeably increased the number of microglia/macrophages in the injured brain. Intracerebral transplantation of BMSCs significantly enhanced the number of astrocytes and in a lesser degree caused changes in the number of microglia/macrophages [165]. Furthermore, transplanted BMSCs reduce the thickness of the glial scar wall and the number of Nogo-A-positive cells along the scar border. BMSCs can also reduce neuron-specific enolase expression, increase reactive astrocyte density, and increase the number of proliferating cells in the SVZ following cerebral ischemia [166]. Hofstetter and colleagues transplanted BMSCs into the injured cord and found that the engrafted BMSCs were tightly associated with longitudinally arranged immature astrocytes and formed bundles bridging the epicenter of the injury [167]. Additionally, BMSC treatment can increase the number of oligodendrocyte precursor cells along the SVZ in the ischemic hemisphere [129]. BMSCs in the penumbra activate local astrocytes as well as increase GDNF levels in the ischemic hemisphere, which is released by reactive astrocytes to facilitate the self-repair efforts of the brain against ischemic attack [168]. As well, BMSCs can increase BMP2/BMP4 expression in ischemic astrocytes. These changes enhance SVZ progenitor cell gliogenesis by activating relevant signaling pathways.

BMSC-stimulated signaling of endogenous astrocytes may alter the ischemic environment, promoting remodeling of the brain after stroke [169]. BMSCs produce growth factors, activate the Akt pathway, and increase the survival of oligodendrocytes (OLG). BMSCs reduce p75 and caspase 3 expressions in OLGs, which lead to decreased OLG apoptosis. BMSCs participate in OLG protection that may occur with promoting growth factors/PI3K/Akt and inhibiting the p75/caspase pathways [170]. In addition, BMSCs have the ability to maintain the resting phenotype of microglia or to control microglial activation through their production of several factors [164]. Transplanted BMSCs could survive and improve neuronal behavior in rats with TBI and mediate neuroprotection and regeneration, which could be associated with the GDNF regulating apoptosis signals through BAX and BAD [171].

4.9 Conclusion

BMSC transplantation is a promising therapeutic strategy for nervous system diseases with unique advantages. Many obstacles associated with medical ethics and immunological rejection can be overcome, while the capability of self-renewal and multidirectional differentiation of BMSCs can be retained after transplantation. Through the interaction with other cells, BMSCs benefit the neurological dysfunction in either direct or indirect methods. Importantly, BMSCs-mediated immunomodulation may be an active component in inflammation modulation, tolerance induction, and reduction of transplantation complications such as rejection and GVHD, even though the complete mechanisms of immune modulation by BMSCs require further investigations. Furthermore, modification of BMSCs with neuroprotective factor- or neurotrophic factor-encoding genes enhances their therapeutic efficacies. Nevertheless, the clinical application of BMSC transplantation is limited, and safety issues must be considered. The fundamental properties of BMSCs and their potential for short- and long-term toxicity need to be determined before they can be widely used in clinical practice.

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Chapter 5
Mesenchymal Stem Cells for Stroke Therapy

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Abstract Mesenchymal stem/stromal cells (MSCs) from different sources have been tested in experimental ischemic stroke and been proved effective in many studies. More understanding of the underlying mechanisms for MSC therapy is essential for improving the therapeutic efficacy and ameliorating or avoiding side effects. In this chapter, we reviewed progress in the field that includes the following aspects: (1) the neurotrophic and immunomodulatory functions of MSCs, the two main mechanisms through which MSCs exert effects in stroke treatment; (2) the aging and senescence of MSCs and their influence on outcome; (3) how autologous MSCs derived from diseased patients may affect the therapeutic effects; (4) different sources of MSCs and comparison of their effects in therapy; and (5) time of transplantation and the delivery methods.

Keywords Mesenchymal stem/stromal cells • Stroke • Transplantation • Neurotrophic • Immunomodulatory

Although a large number of preclinical and clinical tests/trials have been performed over the past 50 years, there are few drugs that have been proved effective for protection or repair of the damaged central nervous system (CNS) following ischemic stroke [1]. However, recent decades have seen a fast development in cell therapy which may potentially promote functional recovery from various CNS disorders, including ischemic stroke.

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A variety of cell types have been tested as cell sources of transplantation in animal models of CNS disorders, including embryonic stem (ES) cells, neural stem cells (NSCs), induced pluripotent stem (iPS) cells, and mesenchymal stem/stromal cells (MSCs). Accumulating evidence shows that MSCs may be a promising choice in tissue engineering and regenerative medicine due to their immunosuppressive and anti-inflammatory properties. Of these, bone marrow mesenchymal stem/stromal cells (BM-MSCs) have unique advantages in that they can be harvested from patients without posing ethical or immunological problems [2].

Basic and preclinical data support the translation of MSC therapy to clinical trials. The procedures of ex vivo expansion and transplantation of autologous MSCs are safe and well tolerated [3]. Stroke patients treated with autologous MSCs through an intravenous delivery route show improved functional recovery [4, 5]. MSC administration starting 24 h after stroke via different routes, such as intracerebral, intravenous, or intra-arterial, all promote functional outcome after stroke [6, 7]. In addition, delayed treatment of stroke with MSCs at 7 days or at 1 month after stroke onset also increases brain plasticity and improves long-term functional outcome [6, 8, 9].

Although MSCs are being tested in phase II clinical trials, many problems remain to be resolved, such as their precise mechanisms of action, the effects of long-term culture and aging on the functions of MSCs, the optimal delivery routes and time, and so on.

5.1 Mechanisms of the Neurorestorative Effect of MSCs in Stroke

MSCs consist of heterogeneous cell populations and may protect and repair the damaged CNS through multiple mechanisms [10]. Some early studies proposed a cell replacement mechanism for MSCs in the CNS; other studies suggested that MSCs may rescue neurons and promote the proliferation and maturation of local neural precursors through release of trophic factors. MSCs also have anti-inflammatory and immunomodulatory effects in periphery and the brain, providing a neuroprotective microenvironment.

5.1.1 Replacement May Not Be the Main Mechanism for Recovery

MSCs have a tendency to home to damaged tissue sites. When MSCs are systemically administered to humans and animals, they migrate to damaged tissue sites which are normally associated with inflammation [11]. In the context of stroke, MSCs are trapped in the lungs, spleen, and other organs outside the brain when administered systemically [12, 13]; only a small number may reach the ischemic sites.

BM-MSCs can be administrated into rats after stroke via intra-arterial, intravenous, and intraperitoneal routes. The dynamic distribution of infused MSCs was

monitored by real-time imaging. After both intra-arterial and intravenous infusion, MSCs were detected in the lungs, spleen, liver, and other organs [14]. Part of infused MSCs were attracted by injury-induced chemokines and migrated toward the ischemic border [15–17]. Only a small portion (<5 %) were detected in brain parenchyma 14 days after transplantation. More than 80 % of MSCs that had reached the brain were localized to the ipsilateral hemisphere, the majority of which congregated in the ischemic border zones [18].

Controversy still exists regarding whether engrafted MSCs can replace damaged neurons. Some studies indicate that transplanted BM-MSCs can express proteins specific to neurons, astrocytes, and endothelial cells in the peri-infarct areas [19–22]. However, very few transplanted cells are found in the brain, and only a small percentage of these cells express neural markers [23–25]. In addition, when MSCs are administered 24 h after stroke, functional outcome is significantly improved from 7 days after treatment [6]. This benefit is not likely attributed to the very few MSCs differentiating into brain cells. Up to now, it is generally accepted that even though MSCs may trans-differentiate and replace some damaged neurons, it is not the main mechanism of therapeutic action.

In addition, some studies suggest that migration of transplanted MSCs into the brain is of some importance for the therapeutic effects. Compared with intravenous transplantation of MSCs, intra-arterial transplantation following ischemic stroke in rats results in smaller infarct volumes, better behavioral recovery, and a larger number of surviving cells in the brain. It is possible that by intravenous delivery, more cells are trapped in the peripheral organs, especially in the lungs, spleen, and lymphoid. The relatively higher number of cells that migrate to the injury sites through intra-arterial delivery route may presumably account for the observed therapeutic effects [26]. Furthermore, it looks like that the brain areas where MSCs migrate to also matter. A study reports that MSCs that have migrated to ischemic cortical regions, which are in charge of the motor functions of animals, act better than those that have migrated to the striatum, in facilitating the recovery of neurological functions [27, 28].

Other than the slim possibility of trans-differentiation into neurons or glia, the mechanisms underlying the beneficial effects from cells that have infiltrated into the brain may be closely related to the neurotrophic and immunomodulatory effects.

5.1.2 Neurotrophic Effects from MSCs

5.1.2.1 MSCs Secrete Trophic Factors

That functional recovery is detected often with very few transplanted MSCs surviving in the brain suggests that the cells may exert a persistent effect. MSCs may support the survival of the endogenous brain cells through paracrine production of soluble factors, including vascular endothelial growth factor (VEGF), Basic fibroblast growth factor (bFGF), glial-derived neurotrophic factor (GDNF), epidermal growth factor (EGF), brain-derived neurotrophic factor (BDNF), etc. [29, 30].

These growth factors have shown effects in promoting cell proliferation/migration and enhancing angiogenesis and vascular stabilization in the ischemic boundary [29–32]. In addition to cytokines, MSCs also produce factors responsible for extracellular matrix remodeling, such as collagen, matrix metalloproteinases (MMPs), and the tissue-derived inhibitors. With the soluble trophic factors, MSCs can prevent neurons from apoptosis, support angiogenesis/neurogenesis, and enhance synaptic plasticity, therefore promoting functional recovery after stroke [33, 34].

MSCs are also capable of producing certain cytokines to ameliorate inflammation-related injury. It has been documented that MSCs can interact with immune cells and produce more than ten soluble cytokines, such as TNF-stimulated gene-6 (TSG-6), hepatocyte growth factor (HGF), transforming growth factor (TGF)-β, prostaglandin (PG) E2, interleukin-(IL-)6, IL-10, IL-1 receptor antagonist, inducible nitric oxide synthase (iNOS), indoleamine 2,3-dioxygenase (IDO), galectin-1, and human leukocyte antigen (HLA)-G [35]. MSCs protect host cells from oxygen free radicals through secreting antioxidants and antiapoptotic molecules.

5.1.2.2 MSCs Induce Host to Secrete "Healing" Factors

As only a small portion (<5 %) of injected BM-MSCs can be detected in brain parenchyma 14 days after transplantation [18], it is unlikely that bioactive factors released by local MSCs per se are the only and direct contributors for the improvement of neurological functions. Intravenously administered cells may not even need to enter the brain to elicit an effect but rather can act in the periphery to increase trophic factor expression in the brain [8]. MSCs can secrete potent combinations of trophic factors to evoke responses from resident cells in the host, thus amplifying the endogenous levels of trophic factors in the brain.

Nowadays only limited knowledge is available about how MSCs modulate the molecular composition of the periphery system and CNS and how to induce the host cells to secrete neurotrophic factors.

In the CNS, astrocyte is one of the cell types that could be influenced by transplanted MSCs. Whether local reactive astrocytes act as the mediators between the limited number of MSCs infiltrating into the brain and the functional recovery has been studied. Being the most abundant cells in the brain, astrocytes provide many supportive activities essential for neuronal functions under physiological conditions [36, 37]. After various insults, including ischemia, local astrocytes are activated and undergo cellular hypertrophy. Previous studies show that MSCs can partially rescue damaged astrocytes, enhance astrocyte activation at the ischemic sites [38, 39], and induce astrocytic production of trophic factors like BDNF, bFGF, NGF, GDNF, and bone morphogenetic protein (BMP)2/4 [40–45]. The increased level of neurotrophins and growth factors produced directly from transplanted MSCs as well as those from evoked host cells could enhance the repair of injured cells around the ischemic tissue by increasing their viability and proliferation or by decreasing apoptosis of the injured neural cells.

In addition to astrocytes, brain endothelial cells also play an important role in MSC treatment of stroke. MSCs cocultured with mouse brain endothelial cells (MBECs) significantly increased MBEC expression of Angiopoietin-1/Tie2. MSCs treatment of stroke rats increases expression of Angiopoietin-1/Tie2 in brain endothelial cells as well as promotes angiogenesis and vascular stabilization [46].

Whether other cell types, such as neurons and microglia/macrophages, are involved requires further study. If so, the detailed mechanisms underlying the interaction of these cells with MSCs remain to be addressed.

Interestingly, the neurotrophic factors produced by host cells (triggered by infused MSCs) might be of more importance than those directly from the MSCs. When human MSCs (hMSCs) were transplanted into the peripheral circulation of rats, the neurotrophic factors of rat origin, not human, were positively correlated with the therapeutic outcome of rats [47]. It thus may be an efficacious approach to enhance the stimulating effect of infused MSCs to achieve a better outcome.

5.1.3 Immunomodulatory Functions

5.1.3.1 Anti-inflammatory Effects

Following CNS damage, inflammatory responses, which is characterized by pro-inflammatory cytokine release [48], lymphocyte infiltration [49, 50], and microglia activation [51, 52], are rapidly induced in the damaged CNS parenchyma. Clinical and preclinical studies suggest that inflammation has an important impact on stroke outcome and long-term prognosis. More severe neurological deficits were reported in stroke patients with preceding infections. A unique feature of MSCs is the immunomodulatory function, which may account for the beneficial effect in suppressing the pathological processes following stroke.

MSCs can suppress lymphocyte activation in response to allogeneic antigens by releasing immunosuppressive cytokines and factors [53, 54]. MSCs can also shift T-cell response from a T helper (Th)1 to a Th2 phenotype. In addition, MSCs are able to inhibit Th17 cell differentiation and function through cell–cell contact and by PGE2.

MSC-derived PGE2 and TGF-β1 play a central role in the induction of CD4+CD25+FoxP3+Treg cells. Purified Treg cells in coculture with MSCs are able to suppress the proliferation of lymphocytes in response to alloantigen. MSCs do not only regulate Treg cells but are also capable of affecting B cells, natural killer (NK) cells , and dendritic cells (DCs). MSCs have been shown to alter B-cell proliferation/activation, differentiation, and antibody production. MSCs can inhibit the proliferation of NK cells [55, 56], prohibit the differentiation monocytes and $CD34^+$ progenitors into antigen-presenting DCs [57], as well as reduce expression of major histocompatibility complex class II (MHCII), CD40, and CD86 on DCs following maturation induction [34].

Furthermore, MSCs alter the cytokine secretion profile of immune-related cells, such as DCs, naive and effector T cells, and NK cells, to induce an anti-inflammatory or tolerant phenotype. For example, hMSCs can induce mature type 1 DCs to decrease TNFα secretion and mature type 2 DCs to increase IL-10 secretion. MSCs can also regulate TH1, TH2, and NK cells to decrease interferon-γ (IFN-γ) secretion and upregulate IL-4, respectively [58].

Accumulating evidence has confirmed the immunomodulatory effects of MSCs. Yet, the fully depicted molecular details still warrant further effort.

Among the mechanisms by which MSCs suppress the overzealous immune actions in diseases, the suppression of lymphocyte activation in response to allogeneic antigens by releasing immunosuppressive cytokines and factors has been widely documented. Furthermore, the peripheral immune organs, especially the spleen, take part in and play an important role in systemic inflammation after stroke. In this process, the modulatory functions of MSCs on the excessive inflammation and immune reactions after stroke may act through the spleen (will be discussed later).

5.1.3.2 How Do MSCs Suppress T Cells?

It has been a long time ongoing debate with regard to how MSCs suppress T cells. In previous work, several groups reported that MSCs inhibit the proliferation of T cells that are induced by alloantigens and nonspecific mitogens [59]. A number of studies established the concept that both soluble factors and cell–cell contact are required for the suppression [60]. In term of soluble factors, PGE2, IFN-γ, and NO are involved and of great importance.

The seemingly divergent concepts have been summarized and updated in Ren's work [61]. Ren and colleagues used MSCs derived from mice deficient in either the IFN-γ receptor or iNOS and showed that immunosuppression is mediated by INF-γ combined with any one of the pro-inflammatory cytokines, TNFα, IL-1α, or IL-1β. Such pro-inflammatory cytokine combinations lead to the release of chemokines from MSCs. The chemokines attract CXCR3-expressing T cells, and then the MSCs produce NO to suppress the proliferation of T cells. Both IFN-γ and NO are indispensable in exerting the suppressive effect. In addition to the in vitro work as mentioned above, the in vivo work using a mouse graft-versus-host disease (GvHD) model showed that the wild-type MSCs significantly reduce GvHD, but MSCs derived from IFNgammaR1(−/−) or iNOS(−/−) mice failed to prevent GvHD [61].

Further studies confirmed that the key players involved in MSC-mediated immunosuppression include indoleamine 2,3-dioxygenase (IDO) and iNOS. Human and other primate MSCs produce extremely high levels of IDO and very low levels of iNOS, whereas mouse MSCs express abundant iNOS and a low level of IDO. In vivo and in vitro studies showed that IDO plays an indispensable role in MSC-mediated immunosuppression in humans [62].

5.1.3.3 MSCs May Not Always Be Immunosuppressive

Many studies have shown that the immunosuppressive feature of MSCs is useful in treating immune disorders. As an encouraging example, in vitro expanded allogeneic MSCs successfully resolved severe grade IV acute GvHD [63]. However, several other reports showed that the effects of MSCs in GvHD are not always consistent. In some cases, MSC treatment could not prolong graft survival or suppress GvHD in vivo, although the MSCs are able to inhibit lymphocyte proliferation in vitro [64, 65].

These results suggest that the immunomodulatory effects of MSCs are conditional and may be dependent on the disease contexts [63]. As stated above, exposure to IFN-γ combined with any of the pro-inflammatory cytokines, TNFα, IL-1α, or IL-1β, is required to turn MSCs to acquire an immunosuppressive feature. When MSCs are not sufficiently or properly primed, they may become immunopromotive rather to be immunosuppressive. Crop et al. cultured human adipose tissue-derived autologous or allogeneic MSCs with nonactivated peripheral blood mononuclear cells (PBMCs) and found that 7 days of coculture with MSCs significantly increased the proliferation of PBMCs as high as threefold [66]. This immune-promoting effect suggests that, under some conditions, MSCs can enhance immune responses [67]. Exposure to IFN-γ combined with TNFα, IL-1α, or IL-1β is required to induce the immunosuppressive activity. However, when pro-inflammatory cytokines are inadequate to elicit sufficient production of NO or IDO or when iNOS activity is inhibited or genetically ablated, MSCs show an opposite effect and strongly enhance T-cell proliferation in vitro and promote a delayed-type hypersensitivity response in vivo. It seems that NO or IDO acts as a switch in MSC-mediated immunomodulation. Importantly, the dual effects on immune functions with IDO acting as a switch have also been observed in human MSCs [67].

Taken together, activation by pro-inflammatory cytokines is essential to bias MSCs into an immunosuppressive phenotype. If the environment cannot provide sufficient pro-inflammatory cytokines, MSCs may exert a different role and enhance immune responses. To take advantage of the immunosuppressive functions of MSCs, it may be useful to prime MSCs in vitro with IFN-γ and any of the pro-inflammatory cytokines, TNFα, IL-1α, or IL-1β, prior to transplantation.

Based on this theory, it is not difficult to understand that the critical determinants of anti-inflammation therapy for stroke patients are the time window and inflammatory cytokine levels. The levels of inflammatory cytokines in recipients change temporally, and at some time points may be insufficient to elicit the immunosuppressive phenotype of MSCs. Therefore, pretreatment of MSCs with inflammatory cytokines may enhance the therapeutic efficacy. It has been reported that IFN-γ-pretreated MSCs can enhance the therapeutic effect in animal models of acute myocardial ischemia [68] and protect 100% of mice from GvHD-induced death [69]. Nevertheless, the therapeutic effect of pretreated MSCs in cerebral ischemia has not been investigated and warrants further research.

5.1.3.4 PGE2 Is a Key Factor in MSC-Mediated Immunosuppression

It was originally demonstrated by Nemeth et al. that the anti-inflammatory effects of lipopolysaccharides (LPS)- or TNFα-activated MSCs could be attributed to MSCs synthesizing and secreting PGE2 [70]. Others have shown that murine MSCs inhibit local inflammation in experimental arthritis through IL-6-dependent production of PGE2.

PGE2 is a lipid molecule derived from arachidonic acid through cyclooxygenase 1 (COX1) and cyclooxygenase 2 (COX2). These enzymes, and PGE2, are constitutively expressed by MSCs, and their expression increases in an inflammatory environment. For example, in vitro studies showed that MSCs produce more PGE2 when cocultured with ConA-induced T-cell blasts or pro-inflammatory cytokines [71]. PGE2 can turn activated macrophages into a regulatory-like phenotype and is able to inhibit the maturation and functions of monocyte-derived DCs [70]. MSCs can suppress LPS-induced glial activation in organotypic hippocampal slice cultures through secretion of PGE2 [72].

5.1.3.5 TSG-6 Is Another Mediator by Which MSCs Modulate Inflammation

Another important mediator in MSC-mediated immunosuppression, particularly in the spleen, is TNFα-stimulated gene/protein 6 (TSG-6). After settling down in the peripheral organs, mainly in the lungs and spleen, MSCs are stimulated to secret TSG-6 upon exposure to TNFα and other pro-inflammatory cytokines produced by resident immune cells. TSG-6 can reduce nuclear factor-κB (NF-κB) signaling in the resident macrophages as a negative feedback. Human umbilical cord cell treatment of stroke in the acute stage significantly reduces the inflammatory response through TSG-6, resulting in decreased necrotic and apoptotic cell death in the brain and increased motor and cognitive functions [73].

5.1.3.6 Immunomodulation Through the Spleen

Ischemia lesion in the brain, especially a severe ischemia, leads to inflammation not only locally in the brain but also in the peripheral immune organs. Studies have shown that the inflammatory status in the brain affects the periphery and vice versa.

The spleen is one of the organs that play an important role in the periphery after stroke. As the largest immune organ in the body that stores lots of T cells, B cells, NK cells, and monocytes/macrophages, the spleen is able to mobilize the immune cells and cytokines into the blood circulation immediately upon injury [74].

After the initial ischemic attack, the blood–brain barrier (BBB) is compromised. That the vascular endothelial cells express adhesion molecules permits an influx of peripheral immune cells including macrophages, neutrophils, leukocytes, T cells, and B cells into the brain [75]. This influx of peripheral immune cells exacerbates

the local brain inflammatory responses, leading to enhanced neurodegeneration and cell loss. It was reported that the infiltrating immune cells and the increased pro-inflammatory cytokines negatively affect stroke outcome [76].

To confirm that the spleen is the main source of cells that infiltrate into the brain in an MCAO model, carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled splenocytes were traced. The number of labeled splenocytes decreased in the spleen and increased within the circulation at 48 h after MCAO. The labeled cells were found only in the injured hemisphere of MCAO animals and were mainly located in the vasculature. This phenomenon suggests that splenocytes may release factors at the BBB and facilitate immune cell infiltration into the brain [77].

The brain and the peripheral immune organs may communicate at least by two pathways, which are the hypothalamic–pituitary–adrenal axis and the sympathetic nervous system [78].

In the case of severe ischemia, the spleen can be affected through these pathways and release plenty of immune cells and pro-inflammatory cytokines. A significant raise of catecholamine concentration in blood circulation occurs following brain ischemia and activates the α1 adrenergic receptors expressed on the splenic smooth muscle cells which results in spleen contraction. In addition, the spleen size was significantly decreased following MCAO in rats and mice [77, 79] as well as in human stroke patients [80, 81]. Correspondingly, this reduction in spleen size is associated with increased release of immune cells and pro-inflammatory cytokines into the blood, a greater extent of infiltration of leukocytes and monocytes, and a higher level of microglia activation in the brain [77, 82].

Some follow-up studies showed that blockade of the α and β adrenergic receptors on the spleen significantly inhibits the reduction in spleen size and reduces the brain infarct volume [83]. Splenectomy prior to MCAO results in 50–80 % decrease in brain infarct volume, which is accompanied by a significant reduction in the number of infiltrating neutrophils and activated microglia/macrophages in the injured brain [81, 84].

The spleen is a highly vascularized immune organ. A large portion of systemically infused MSCs are trapped in the spleen, which makes the cell–cell contact possible between MSCs and immune cells. In this regard, the delivery of MSCs through the peripheral route and suppression of spleen-involved inflammation may influence stroke outcome and prognosis.

In the acute phase after stroke, transfusion of human umbilical cord blood cells (HUCBCs) not only reverses the reduction in spleen size but also alters the splenic cytokine expression profile from a pro-inflammatory (e.g., TNFα and IL-1β) to an anti-inflammatory (e.g., IL-10) one [79]. Not only in the acute ischemia, recently, Acosta et al. found that by intravenous transplantation of hMSCs at 60 days post stroke, labeled hMSCs preferentially migrated to the spleen and reduced striatal infarct and peri-infarct areas compared with those of sham animals. Treatment with MSCs significantly decreased by 75 % and 60 % major histocompatibility complex II-positive inflammatory cells in gray and white matter and reduced TNFα-positive cell density in the spleen of transplanted stroke animals. About 0.03 % hBM-MSCs survived in the spleen and only 0.0007 % hBM-MSCs survived in the brain. MSC

migration to the spleen, but not to the brain, inversely correlated with inflammation and the infarct and peri-infarct volumes [85].

Another notable issue is the immunosuppression after stroke. The spleen usually decreases in size transiently at 1–4 days following MCAO in rats and recovers to its original size within 7 days [77]. Severe cerebral ischemia leads to prolonged spleen contraction, and if this situation persists, eventually, the storage and even the capacity to produce immune cells and pro-inflammatory cytokines will be exhausted. Furthermore, due to the feedback regulation of spleen functions after the surge release of splenocytes and pro-inflammatory cytokines, anti-inflammatory cytokines, such as IL-10 expression, is enhanced. Immunosuppression, which is often the cause of infection and death of stroke patients, may explain why in some cases, anti-inflammation therapy after stroke actually exacerbated the outcome [86].

It seems that the intravenous infusion of MSCs may suppress the "over-activated" inflammation and immune reaction in the spleen, reduce the influx of immune cells and pro-inflammatory cytokine into the brain, prevent the exhaustion of immune capacities of the spleen, and eventually avoid the immunosuppression in stroke patients. From this point of view, one of the major functions exerted by systemically transplanted MSCs may be acting as a buffer in the process of inflammatory events following stroke attack and preserving the normal immune functions of the spleen.

Another interesting notion is that the immunosuppressive effect of cell therapy is not restricted to MSCs. Other cell types, such as neural stem cells [87], hematopoietic stem cells [88], bone marrow mononuclear cells [89], and umbilical cord blood cells [90], all have shown some immunomodulatory functions in stroke models. Among these cell types, at least neural stem cells have been reported to have an immunosuppressive ability through the spleen [87].

5.1.3.7 Modulation of Microglia Activation in the Brain

The brain is an immune privileged organ separated from the periphery by BBB, which seals the brain off from toxins, pathogens, and the circulating immune cells. However, upon stroke attack, cerebral ischemia induces a robust neuroinflammatory response that includes marked changes in a variety of endogenous CNS cell types, as well as an influx of immune cells from the peripheral blood circulation. Among these cells, microglia are the main resident immune cells in the brain, which originate from the peripheral monocytes/macrophages during embryonic development [91].

Microglia maintain a constant survey of the immune status in the brain. Microglia can engulf pathogens, misfolded proteins, and scavenge dead cells in the event of infection, ischemia, and neurodegeneration [92, 93]. Microglia also clear away synapses that have been damaged by injuries and, under some conditions, even trim off weak connections/synapses between neurons [94]. Upon activation in acute ischemia, microglia contribute to the injury by the release of pro-inflammatory cytotoxins and other inflammatory mediators, such as TNFα, IL-1β, NO, and reactive oxygen species, which are the direct effectors of cerebral injury, and take part in the

chronic ischemic injury by continuously maintaining the upregulation of these pro-inflammatory cytotoxins and other inflammatory mediators. Meanwhile, microglia may modulate inflammatory responses by releasing anti-inflammatory cytokines, protect neurons by releasing growth factors, and improve endogenous regeneration by clearing debris, and so on [95, 96].

According to the above two opposing status, microglia can be categorized into M1, which is a pro-inflammatory, and M2, an alternative anti-inflammatory phenotype. M1 microglia produce pro-inflammatory mediators such as TNFα, IL-1β, and IFN-γ. M1 microglia express CD80, CD86, and MHC class II on the cell membrane and can present antigens to T cells [97]. M2 microglia secrete anti-inflammatory mediators such as IL-10, TGF-β, IL-4, and IL-13, as well as various neurotrophic factors, such as insulin-like growth factor-1 (IGF-1). M2 microglia is regarded as "healing cells" that contribute to recovery after brain damage [98]. In contrast, M1 microglia tends to induce neuronal cell death more readily than M2 microglia. The M2 phenotype microglia are dominant in both resident microglia and newly recruited macrophages at early stages in ischemic stroke. At 24 h after ischemic attack, Ym-1 and CD206, markers for the M2 phenotype, are found in the ischemic core [99]. The expression of M2 markers is highest at 5 days after insult and start to taper off after 14 days [98], suggesting that microglia/macrophages participate in tissue repair in the ischemic core [100]. However, the M2 phenotype gradually transforms to M1 phenotype in the peri-infarct areas, which shows an exacerbating effect on neuronal death. Therefore, stroke therapies may need to be shifted from a strategy of simply suppressing microglia toward balancing the beneficial and detrimental effects of microglia [96].

MSCs are capable of increasing the mRNA and protein expression of M2 markers on microglia in the coculture system [101], and MSCs have been proven capable of inhibiting the shift of microglia from M2 to M1 or prompting the transition from M1 to M2 in different disease models, such as wound, lung disease, heart disease, renal disease, spinal cord injury, and brain trauma [102]. The pathological processes of some of these diseases are to some degree similar to stroke; therefore the available published data support the hypothesis that MSCs may be competent in modulating the functions of microglia in the ischemic brain.

5.2 Senescence of MSCs

Adult MSCs are a valuable resource for autologous and allogeneic cell therapies. However, patients suffering from stroke are often of advanced age. In light of this, the effects of the aged milieu on MSCs and the intrinsic aging of MSC in vivo are important questions to address. Furthermore, MSCs may require in vitro expansion before use, and their aging in vitro is thus also an important issue.

Compared to younger people, the extracellular microenvironment in older people may present with a more pro-inflammatory characteristics, rendering the aged milieu a more hostile environment for adult stem cells [103]. It has been shown that

the functions of adult stem cells are suppressed in aged mice, suggesting a causal relationship between stem cell aging and organismic aging [104, 105].

To evaluate the correlation of age and the functional properties of MSCs, children and adult MSCs were compared. A shorter population doubling (PD) time and a higher colony-forming unit–fibroblast (CFU-F) count were found in children MSCs as compared with those of adult MSCs. The telomere length was also significantly greater in children MSCs vs. adult MSCs. These data suggest that children's BM-derived MSCs could be a more advantageous source for cell therapy [106].

Similar results were reported in an independent study [107], in which 17 BM samples were collected from young adult donors and 8 from pediatric donors. MSCs isolated from the two groups showed no morphological differences, while their cell growth was strictly related to the donor's age. The MSCs isolated from pediatric donors reached a cumulative population doubling times almost twice as high as MSCs isolated from young adult donors after 112 days [107]. The authors analyzed the surface marker expression from the two groups and no obvious difference was observed. Neither chromosomal alteration nor evidence of cellular senescence was observed in all the analyzed samples.

Meanwhile, accumulating evidence shows that MSCs undergo senescence itself in vitro and in vivo.

Kim et al. reported the replicative senescence of MSCs in vitro. The human MSCs (hMSCs) were cultured for more than 60 population doublings. In the long-term cultured hMSCs over PD 30, the adipogenic and osteogenic differentiation potentials were significantly reduced. Telomere length was shortened although the telomerase activity was unchanged. Oncogenic gene expression decreased in proportion as the PD number increased, and no tumor formation was observed when the long-term cultured hMSCs were injected into nude mice [108].

In another study using human tonsil-derived MSCs, Yu and colleagues investigated the MSCs for up to passage number 15. They found no alterations in the expression of MSC-specific surface markers, CD14, CD34, CD45, CD73, and CD90. However, the expression of CD146, recently identified as another MSC marker, dramatically decreased from ~23 % at passage 3 to ~1 % at passage 15. The average doubling time increased significantly from ~38 h at passage 10 to ~46 h at passage 15. From passage 10, the cell size increased slightly and SA-β-gal staining was evident. Also, the osteogenic differentiation ability increased up to passage 10 and decreased thereafter. However, the adipogenic and chondrogenic differentiation potentials decreased since the start of passaging in vitro [109].

The molecular mechanisms underlying MSC senescence have been explored. MSCs obtained from systemic lupus erythematosus (SLE) patients exhibit accelerated functional decline as part of the SLE phenotype. In these cells, expression of the senescence-related gene p16 is increased [110]. In other studies, senescence-related genes including p16, p21, and p53 were increased in long-term cultured MSCs [111–113].

Another mechanism that may be involved in MSC senescence is epigenetic regulation. Schellenberg analyzed the functional, genetic, and epigenetic status of long-term cultured MSCs [114]. Although chromosomal aberrations were not detected

by karyotyping and single-nucleotide polymorphism (SNP) microarrays, highly consistent senescence-associated modifications at specific CpG sites were noted, such as trimethylation of H3K9, H3K27, and EZH2 targets. It seems that culture expansion of MSCs has profound functional implications, which may be hardly reflected by genomic instability but is associated with highly reproducible DNA methylation changes and repressive histone marks.

While the use of MSCs as cellular therapies for different clinical problems is still under investigation, it does appear that aged MSCs generally perform less effectively than their younger counterparts in various disease models [115, 116]. Many age-related changes in MSCs seem to be in agreement with this hypothesis. Collectively, the general age-associated changes may include MSC adhesion, migration, resistance to oxidative stress, and cytokine secretion. These changes could have important implications in tissue healing mediated by MSCs, through their homing, migration, and paracrine stimulation of other cells [115, 117, 118]. For example, MSCs isolated from aged rats showed a significantly reduced antioxidant and migration capacity [115, 119], as well as a decreased in vitro wound healing function [120]. Furthermore, MSCs obtained from 6-day-old to 6-week-old mice are significantly more adhesive than those from 1-year-old mice [121], while MSCs from 3-month-old mice secrete an extracellular matrix that is more proliferation- and osteogenesis-promoting than that from older mice [117].

However, not all studies support the notion that MSCs are affected by aging. For example, it was found that the ability of hMSCs to suppress proliferation of activated allogeneic T cells in vitro is not affected by biological age [122].

Despite the results discussed above, the information about the influence of aging is limited with regard to the roles of MSCs in stroke therapy. Li et al. found that ischemia-induced neurogenesis is enhanced to a higher extent by intravenous administration of hMSCs of early passage than those of later passage. The effects may be related to passage-dependent neurotrophic capacities of MSCs [123].

It is noteworthy that aging of MSCs in vitro vs. in vivo may be different. MSCs cultured in a dish may experience certain level of stress and lack the interaction with the microenvironment in a tissue. In addition, although less immunogenic, the interaction with immune cells in vivo may exert some pressure and screening effect on MSCs and influence the aging process and physiological roles of MSCs in vivo. Another thing that needs to be kept in mind is that MSCs are not a homogeneous population; it is possible that the effects observed by aging are only due to changes in a subpopulation of the MSCs. Further studies are required to address these issues.

The above findings suggest that genome test, at least karyotyping assay before cellular therapy, should be performed during the ex vivo culture of hMSCs, particularly when an extended propagation period is required.

5.3 Features of MSCs from Individuals with Diseases

Compared with allogeneic cell therapy, autologous MSC transplantation has certain merits, such as less risk of contamination, fewer ethical issues, and ease of acceptance by patients. However, since autologous MSCs are mostly derived from diseased people, it warrants a close examination whether the disease state affects the properties of MSCs.

Diabetes is a common disease. In type two diabetic and prediabetic patients, advanced glycation end products (AGEs) are normally present at an elevated level, which may suppress the proliferation, induce apoptosis, and increase the intracellular reactive oxygen species production in the MSCs [124].

Another disease condition/indication that has been investigated is heart failure. Dmitrieva and colleagues found that BM-MSCs from the heart failure patients demonstrate an early reduction in the proliferative activity and change of gene expression profiles that favor a pro-fibrotic phenotype of MSCs, which makes these cells less effective for therapeutic applications [125]. However, other study also found that treatment of stroke with BM-MSCs derived from brain ischemic stroke rats (isch-BMSCs) significantly improves functional outcome after stroke and shows better therapeutic effects than BM-MSCs derived from normal rats (Nor-BMSCs) [126]. Isch-BMSCs are superior to Nor-BMSCs for the neurorestorative treatment of stroke, which may be mediated by the enhanced trophic factor and angiogenic characteristics of Isch-BMSCs [126].

Cancer also influences BM-MSCs function. BM-MSCs from multiple myeloma patients have an increased expression of senescence-associated β-galactosidase, increased cell size, reduced proliferation capacity, and an aberrant secreting profile. In addition, multiple myeloma patient BM-MSCs more efficiently support the growth of $CD34^+$ cells, suggesting that not only BM-MSCs are altered by the disease state but that these cells may actively contribute to the disease progression [127].

Irradiation is an often chosen treatment for cancer patients, and it would be useful to learn whether irradiation affects the functions of BM-MSCs. Cmielova et al. studied the characteristics of BM-MSCs derived from patients who had received up to 20 Gy ironizing irradiation. These BM-MSCs showed significantly reduced proliferation but no obvious change in cell survival. Activation of p53 was detected from the first day of irradiation and remained elevated to day 13. The cell cycle was arrested in G2 phase, in agreement with the upregulated expression of cyclin-dependent kinases inhibitor 1A (p21Cip1/Waf1). Instead of apoptosis, the authors detected hallmarks of stress-induced premature senescence: increase in cyclin-dependent kinases inhibitor 2A (p16INK4a) and increased activity of senescence-associated β-galactosidase [128].

In another study, Li et al. examined the properties of BM-MSCs isolated from patients suffering from systemic lupus erythematosus (SLE), an autoimmune disease characterized by a mistaken immune attack on the patient's own internal organs/tissues. The MSCs exhibited impaired proliferation, differentiation, cytokine

secretion profiles, and aberrant immunomodulatory functions. An increased frequency of apoptosis and aging in SLE BM-MSCs was also observed compared with those of normal controls. Moreover, intracellular reactive oxygen species levels of SLE BM-MSCs were higher than those of normal controls [110].

From the above studies, it seems that, despite the advantages associated with autologous transplantations, application of syngeneic MSCs from individuals suffering from certain diseases may not be the best choice. In such cases, allogeneic MSCs from healthy donors may be considered.

5.4 Different Sources of MSCs

Finding an appropriate and convenient cell source is a key step for cell therapy and tissue engineering.

Bone marrow has been one of the first reported cell sources for MSCs [129] and BM-MSCs remain a good choice for autologous transplantation [130]. However, several caveats exist regarding the clinical application of BM-MSCs. Firstly, there are only one MSC in around 10,000 cells in the bone marrow, giving rise to a relative low yield of MSCs; secondly, MSCs decrease in the proliferation and differentiation capacities with age, which may be a particular issue for old patients; thirdly, the isolation procedure is painful, and with allogeneic transplantation of BM-MSCs, the risk of viral exposure cannot be completely avoided.

Given the above aspects associated with BM-MSCs, researchers and clinicians have searched for alternative sources of MSCs. MSCs can also be extracted from adipose tissues (AT) and embryo-related tissues, such as umbilical cord (UC), chorionic plate (CP), and placenta.

Among the different sources, adipose tissue and bone marrow remain the most accessible sources to derive MSCs, and both can be used for autologous transplantation purposes. Compared with BM-MSCs, AT-MSCs are less invasive and less expensive to obtain and show a higher proliferative capacity with shorter population doubling times. But both AT-MSCs and BM-MSCs undergo senescence after certain number of passages in vitro. Compared to AT-MSCs and BM-MSCs, embryo-related MSCs (UC-MSCs, CP-MSCs, and placenta MSCs (P-MSCs)) are usually used for allogeneic purposes. Originated from tissues of early developmental stage, embryo-related MSCs retain a primitive stemness feature and possess a good proliferative capacity. Many studies compared the proliferative capacity between BM-MSCs and UC-MSCs and found that UC-MSCs show a much higher proliferative activity and no sign of senescence even after an extensive culture [131, 132]. Conconi et al. cultured UC-MSC over 16 serial passages and found no aberration in cell morphology or senescence. Mitchell et al. cultured porcine UC-MSC for more than 80 doubling times without detecting decrease in the proliferative capacity [133].

In terms of the immunosuppressive ability, Najar et al. observed a potent and dose-dependent inhibition of lymphocyte proliferation by these MSCs, regardless

of the stimuli used to activate T cells. UC- and AT-MSCs display an inhibitory effect higher than that with BM-MSCs [134]. Ribeiro et al. compared the immunomodulatory abilities between human UC-, AT-, and BM-MSCs. They found that MSCs derived from all three tissues are able to inhibit the activation of $CD4^+$, $CD8^+$ T cells, and $CD56^{dim}$ NK cells, wherein AT-MSCs show a stronger inhibitory effect. Moreover, AT-MSCs block the T-cell activation process at an earlier phase than BM- or UCM-MSCs do, yielding a greater proportion of T cells in the nonactivated state [135].

Castro-Manrreza and colleagues compared BM-, UC-, and P-MSCs in their immunosuppressive properties. Except for P-MSCs, BM-MSCs and UCB-MSCs significantly inhibit the proliferation of both $CD4^+$- and $CD8^+$-activated T cells as well as increase the generation of $CD4^+CD25^+CTLA4^+$ Treg populations [136]. Therefore, in addition to BM-MSCs, at least UC-MSCs and AT-MSCs may be potent and reliable candidates for future therapeutic applications. However, which one is the best is not fully known and more work is needed to address this issue.

5.5 Multi- or Single Infusion of MSCs?

Many studies have demonstrated that systemic injection of hMSCs prepared from bone marrow or other sources have therapeutic benefits in rat ischemia models [47]. Yet, there is still a shortage of data comparing the therapeutic effects between a single and multiple injections of MSCs in stroke models.

Multiple infusions at various sites and times may lead to a cumulative response/effect. In the mouse GvHD model, multiple administrations of MSCs are often utilized to sustain and prolong their inhibitory effect [54]. Yet it is not clear whether repeated intravenous MSC administration would give a better outcome for stroke treatment.

To test this, Omori et al. used the MCAO model of rats, and infused hMSCs intravenously at a single time point 6 h post-ischemia (low and high cell doses) or at various multiple time points after MCAO. Ischemia lesion volume was reduced in all hMSC cell injection groups as compared to serum alone injection group. However, the greatest therapeutic benefit was achieved following a single high cell dose injection at 6 h post-ischemia rather than multiple lower cell infusions over multiple time points [137].

In another study, baboons were used to test whether allogeneic MSCs would elicit immune responses. The baboons were injected intravenously with MSCs (5×10^6/kg) followed by intramuscular injections of MSCs (5×10^6/kg) 6 weeks later from the same or a different donor. Almost 30 % of the animals produced alloantibodies that reacted with MSCs; but the host T-cell responses to donor alloantigens did not suppress the overall T-cell response in the host. This is the evidence showing that multiple administrations of high doses of allogeneic MSCs can induce alloreactive immune responses without compromising the overall immune system in nonhuman primates [138].

In a separate study, Isakova et al. found that even one-time intracranial administration of allogeneic MSCs can evoke immune reactions in the host rhesus macaques. In animals administered with allogeneic but not autologous MSCs, allo-recognition and allo-specific antibodies were detected. However, secondary antigen challenge failed to elicit a measurable response in allogeneic recipients. The authors indicated that although the behavior testing did not reveal significant changes related to transplanted MSCs at any doses, allogeneic MSCs were weakly immunogenic when allotransplanted in rhesus macaques, and this negatively influenced the MSC engraftment level and duration [139].

The two nonhuman primate experiments were performed using naive animals rather than using cerebral ischemia models. There is limited information on the immune safety and therapeutic efficacy of repeated intravenous injections of allogeneic MSCs in stroke models or patients. The influence of a single or multiple injections of MSCs, especially MSCs from different allogeneic donors, on the immune responses of the host should be taken into consideration in the clinical trials.

5.6 Time of Transplantation and Delivery Routes

Theoretically, the optimal timing and routes of cell delivery depend on the mechanisms of action of MSCs and the brain environment. As discussed above, the therapeutic mechanisms of MSCs are mainly related to the migration of transplanted cells, the modulation of inflammation/immune functions in the CNS and peripheral immune organs, and the neurotrophic effects from MSCs.

In literature, a wide range of intervals post-ischemia have been reported. Many of them demonstrated that functional recovery can be achieved by cell therapy within the first 2 days after ischemia. There are also studies showing a cell-enhanced recovery where cells are delivered up to 1 month post-ischemia. The optimal time window in the clinical setting has yet to be determined [85, 137].

The brain environment changes dynamically during the acute and subacute phases after stroke onset. In the acute phase (0–2 days after stroke in rodents), there is an increase in inflammation/immune reactions, represented by release of excitatory amino acids, pro-inflammatory cytokines, reactive oxygen species, etc. This is followed by a subacute phase (several days) during which the inflammation/immune reactions decrease continuously. Then the animals go into the chronic phase during which attempted endogenous brain repair may last several weeks to months.

If the cells are intended to be deployed into brain parenchyma, for example, by the intracerebral stereotaxic transplantation, survival of the engrafted cells would be critical; in this case, delivery at the subacute or later stages may be a good choice; at which time point, the inflammation in the brain has been ameliorated. As far as the inflammatory milieu is concerned, direct transplantation into the penumbra, where the microenvironment is not as hostile as the ischemia core, may enhance the survival of MSCs that in turn could modulate the ongoing inflammation and provide neurotrophic protection to neurons.

If the immunosuppressive function of MSCs is desired, exposure to inflammatory cytokines is required to induce the immunosuppressive phenotype of MSCs and should be taken into account. At the acute stage after stoke, inflammatory cytokines are produced at peak levels, at which time infusion of MSCs may lead to a good outcome for immunosuppression.

Other treatment strategies, no matter aiming at neuroprotection or at enhancing the endogenous neuroregeneration, are related to the neurotrophic mechanisms. The knowledge is limited about what factors regulate the secretion of neurotrophic factors from MSCs and how MSCs stimulate the host cells to participate in neuroprotection and neurorestoration. Delivery at acute or later stages may be both beneficial in these conditions; but an earlier intervention may be desirable since it may be good for the neurotrophic protection to start as early as possible.

Nevertheless, thorough data concerning the optimal timing of intervention are still lacking and the abovementioned hypotheses need to be further tested.

In terms of the delivery routes, MSCs can be transplanted intracerebrally, intravenously, or intra-arterially.

The delivery routes most likely affect the trafficking of MSCs to the target organs. Intracerebral injection is the most efficient method for delivering MSCs to the damaged brain tissue, but other methods also have their merits.

Intravenous and intra-arterial injections are the two widely used approaches of systemic administration. As stated above, by intravenous injection of MSCs, MSCs tend to migrate to the peripheral immune organs, including the lung, spleen, liver, and thymus, whereas the intra-arterial method is favorable for the aggregation of MSCs in the injured brain areas.

There are a limited number of studies that directly compared the therapeutic effects of different delivery routes under the same conditions.

Both intravenous and intra-arterial transplantation of bone marrow mononuclear cells (BM-MNC) and BMSCs improved functional recovery and reduced lesion size in stroke rats as compared with saline treatment [6, 7, 28]. However, no significant differences between intravenous and intra-arterial groups were observed.

Savetz et al. found that intra-arterial delivery led to more BM-MNCs in the peri-infarct area at 1 and 6 h after ischemic attack; but unexpectedly, the presence of a larger number of cells in the brain did not correlate with functional improvement or tissue repair as previously suggested by others. The authors suggested that, once reaching the quantity threshold, more cells present in the brain may not lead to a greater recovery [28].

Compared to BM-MNCs, delivery of MSCs through different routes may be a different story. MSCs are much larger than MNCs in size and will be trapped to a higher extent in spleen and lymphoid organs after being delivered intravenously. Accordingly, intra-arterial infusion of MSCs will give rise to more cells in the brain. This raises an interesting question about whether more MSCs in the spleen or in the brain would be more beneficial for stroke recovery. This comparison involves different mechanisms of action exerted by MSCs through the spleen or brain, and such effects may be time and cell dose dependent. Each delivery route may have multiple

therapeutic effects at both the CNS and the peripheral organs. The collective outcome of cell therapy is thus the sum of these therapeutic effects.

Nevertheless, which route of administration is the best for stroke treatment, especially for clinical use, is yet not clear. The therapeutic effects of MSCs at different sites and time points require further studies.

5.7 Conclusion and Perspectives

MSCs can be isolated from different tissues and consist of a heterogeneous cell population. Normally, MSCs need to be passaged a few times in vitro before use. After transplantation through certain routes, MSCs migrate and distribute at different areas, and part of them may reach the injured sites. The microenvironments at different sites vary and change dynamically, and MSCs interact with this complex microenvironment through different mechanisms, presenting with an immunosuppressive, immunopromotive, and/or neurotrophic phenotype, dependent on the specific context.

Given the complexity involved in cell therapy for stroke, an optimal treatment strategy has yet to be worked out. Nevertheless, an increasing body of evidence suggests that using MSCs to treat stroke is safe and to some degree might be efficacious. Compared to conventional drug treatment, cell therapy using MSCs certainly has its advantages. However, to maximize the efficacy with MSCs, the treatment strategy may need to be individualized, taking into consideration at least the disease stages of the patients. In perspective, the stroke patients can be roughly categorized into three groups according to the disease severity, and the treatment strategies may be different accordingly. Still, many questions remain to be addressed in the field.

For example, in severe stroke in which pro-inflammatory cytokines are produced at high levels, infused MSCs may be primed by the cytokines and act in an immunosuppressive way. In this context, will MSC treatment give a better outcome than with anti-inflammatory drugs, in attenuating the acute inflammation and preventing stroke-induced immune suppression?

With regard to stoke of medium severity, it is not yet clear if MSC infusion would dampen or promote the inflammatory damage in the brain, given the condition that the pro-inflammatory cytokine levels may not be high enough to prime MSCs to an immunosuppressive phenotype. If this is the case, will pretreatment with pro-inflammatory cytokines before MSC transplantation improve the outcome?

In the case of mild stroke, in which the inflammation level is not high, how to balance the immunosuppressive, immunopromotive, and the neurotrophic features of transplanted MSCs?

Another closely related question is how to categorize the patients into the three groups of severities. In addition to the clinical symptoms, can we use levels of pro-inflammatory cytokines as a criterion to group the patients? If so, shall we use cytokines in blood or in cerebrospinal fluid as a reference? And which cytokines should be used?

Answering these questions will definitely accelerate the translation of MSC therapy from bench to bedside.

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Chapter 6
Bone Marrow Hematopoietic Stem Cell Therapy in Stroke

Michael Jiang and Ling Wei

Abstract Mammalian bone marrow is host to an active stem cell population with the responsibility of maintaining and replenishing a variety of high turnover cells that are vital for survival, regeneration, and support of the rest of the body. The major outputs of this hematopoietic system are the erythrocytes and platelets of the blood and the granulocytes and macrophages of the immune system. These stem cells also give rise to antibody-producing B-lymphocytes and antibody-producing T-lymphocytes that consume foreign or inflammatory substrates in the body. As all of these substituent cell types have a very limited life span, they must be constantly and consistently replenished. This rapid turnover of up to 100 billion new cells from progenitors forms the hematopoietic system (Mohammadi et al. Int J Stem Cell Res Transplant, 2014 2(02), 59–62). In recent years, bone marrow hematopoietic stem cells have drawn increasing attention for their therapeutic potential used in cell transplantation therapy for neurological disorders including stroke and traumatic brain injury. This review article is to summarize recent progress in basic and preclinical investigations on these cells including hematopoietic and mesenchymal stem cells.

Keywords Bone marrow • Hematopoietic stem cell • Therapy • Stroke

6.1 History of Hematopoietic Research

Hematopoietic stem cells have been studied for more than a half century as a therapeutic approach for patients suffering from compromised immune systems [34]. The hematopoietic system was particularly sensitive to acute radiation with death resulting from either pathogenic invasion or loss of blood due to low levels of platelets or other reasons. In 1952, Lorenz et al. demonstrated that radiation-damaged hematopoietic systems could be rescued by injections of bone marrow cell

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suspensions [36, 64]. This discovery formed the foundation of modern-day treatment for hematopoietic system failure by transplantation of bone marrow hematopoietic stem cells [13].

Loss of cellular regulatory substitutes can occur permanently or temporarily in neurological diseases including ischemic stroke.

The selective vulnerability of the hematopoietic system to low doses of radiation also gained relevance as it was compromised in patients with the advent of chemical or radioactive chemotherapeutic agents that target rapidly dividing cells [54, 57]. The hematopoietic system is comprised of cells that divide rapidly as is the case of many cancer cells. Therefore, drugs and therapies used to treat cancers can cause patients suffering a weakened immune system that requires secondary hematopoietic therapies to restore the immune activity [57]. Much work in the hematopoietic system originated and continues in murine models. Hematopoietic stem cells (HSCs) can be dissected from the yolk sac or the intrabody aorta-gonad-mesonephros (AMG) region of mouse embryos [37, 43]. Surprisingly, adult hematopoietic stem cells are not present during the earliest stages of development but arise after the formation of the hematopoietic system [45].

6.2 Hematopoietic Stem Cells and Stroke

Stroke remains a leading cause of death and disability in the United States and around the world [41]. The current limited availability of effective therapeutics for human stroke patients has inspired multifaceted and increasingly complex approaches to limit ischemic damage and/or enhance post-stroke regeneration. Treatments that can do both have become attractive candidates in stroke research [35]. Among these are various cell-based transplantation therapies which have had demonstrable success in animal models of stroke [3, 5, 67]. The investigation has looked at the effects of transplanting different stem cells and neural progenitor cells, timing of the transplantation, and the delivery method of the desired cells. Cellular therapies have multifaceted effects during both acute and chronic phases of neurological disease. Major cell types currently under investigation including but not limited to induced pluripotent stem (iPS) cells [44], embryonic stem cells [18], bone marrow-derived mesenchymal stem cells [33], and bone marrow hematopoietic stem cells (HSC) [47] offer unique advantages with its own set of drawbacks. Among the various potential cell types used for cell-based stroke therapeutics, HSCs maintain the advantage of being available from autologous donation without risk of graft rejection, low immunoreaction, and self-renewal [1, 15].

Ischemic stroke is characterized by rapid deregulation of normal cellular processes resulting from lack of oxygen. The resulting cell loss due to excitotoxicity and various apoptotic and/or necrotic mechanisms generates a highly toxic environment with compromised access to the hematopoietic system [9]. As such, a temporary ischemic insult can result in a chronic condition within a brain loci that mimics many of the pathological hallmarks characteristic of systemic hematopoietic failure.

Interestingly, ischemic stroke and myocardial infarction can mobilize CD34+ hematopoietic stem cells into blood circulation [47]. Augmenting the body's hematopoietic system by stimulating bone marrow stem cells into the blood and homing to the ischemic region is an attractive means of assuaging stroke pathogenesis during acute and chronic phases of injury.

Brain ischemia is known to trigger immune responses including activation of resident microglia within 24 h [17]. Bone marrow-derived monocytes can enter the brain through the blood-brain barrier near the ischemic region where they also exhibit a microglia phenotype [26]. Both local immune cells and ischemic tissues are known to release cytokines, complement factors, and free radicals that escalate the immune response [30]. While some of these factors have been found to be anti-inflammatory such as IL-10 [20], the general environmental tone is pro-inflammatory. Consequently, both the ischemic region and the peri-infarct regions following stroke harbor a pro-apoptotic and/or pro-necrotic tone. In the days to weeks following ischemia, this toxic environment leads to the expansion of neuronal and glial loss [21].

6.3 Hematopoietic Stem Cell Transplantation

Ischemia results in massive loss of not only neuronal cells but also a host of supportive cell types necessary for maintenance and survival of brain tissue [14]. Together, the loss of these cells represents loss of entire neurovascular units which includes cell types such as endothelial cells, pericytes, astrocytes, and other glial cells [14, 23]. One of the major goals of transplantation of hematopoietic stem cells for stroke is to augment the body's natural ability to supply supportive substituent cell types to the ischemic region. Rather than delivery of a single differentiated cell type to the ischemic region, HSCs have been demonstrated to differentiate into a variety of supportive cell types including astrocytes, oligodendrocytes, neural precursors, macrophages, and microphages [31, 56]. The multipotent characteristic of HSCs makes them a preferable cell therapy without need for combination with other cell types.

Systemic injection of HSCs can be traced using pre-labeling of injected cells to differentiate them from host tissues. Injected GFP-tagged HSCs can be observed in the spleen within the first 24 h of delivery [32]. HSCs typically home to the bone marrow and spleen regardless of delivery methods [24, 50]. A limited number of injected HSCs can be observed in the brain where their most common phenotype is that of microglia-like cells reminiscent of host microglia found in a healthy state [24, 51, 55]. Modification of HSCs by exogenous overexpression of Sca-1, Thy-1, and c-kit in a spinal cord injury model demonstrated differentiation of HSCs into astrocytes, oligodendrocytes, and neuronal precursors [65].

As a cardiovascular disease, ischemic stroke damages both the brain and the circulatory arteries and veins in and around the infarct. Regenerative therapy for ischemic stroke favors approaches that enhance both neurogenesis and angiogenesis.

Toward the latter goal, HSCs include a subset of CD34+ endothelial stem and progenitor cells. HSC therapy via systemic injection or local intracerebral injection after stroke are both known to increase angiogenesis in the peri-infarct region. Along with angiogenesis, these studies also indicate that HSC therapy promotes enhanced functional recovery and reduced infarct size. Specifically, CD34+ cells from HSCs which provide the strongest angiogenic effects can be enriched using immunoselection for acute ischemic stroke [59].

Post-ischemia neurogenesis in the brain stems from two well-studied regions: the subventricular zone and the subgranular zone of the dentate gyrus [22]. While neurogenesis has been well documented to play a role following ischemic stroke in animal models [42, 48], its effect has been minimal with reports of only 0.2 % of neurons being replaced by endogenous neurogenesis [2]. These results can be verified by delivery of CD34 cells from HSC preparations to the post-ischemic brain. Furthermore, consistent with these findings are reports that pro-angiogenic agents such as erythropoietin (EPO) produce similar results as CD34+ HSC delivery [16]. Suppression of endothelial proliferation by endostatin can also directly mask the beneficial effects observed from CD34+ cell delivery [59]. These findings indicate a role for HSC therapy in augmenting endogenous post-stroke regeneration by enhancing neovascularization to support new neurons which have otherwise shown to migrate but quickly die in the toxic environment surrounding stroke [2].

The relationship between vascular support and neuro-regeneration can be linked to effective angiogenic therapies studied in other organs including the liver and thyroid. Supporting regenerating tissues with neovascularization can significantly improve endogenous cell replacement both *in vivo* and *in vitro* [53, 62]. It is hypothesized that improved vascularization may help brain regeneration by providing additional routes for the removal of dead tissue which also doubles as additional migration routes into the ischemic zone. Examination of this hypothesis and various factors that may play a role in promoting the survival of both endogenous and transplanted neuro-progenitors has been extensively investigated including but not limited to fibroblast growth factor 2 [29], platelet-derived growth factor [58], brain-derived neurotrophic factor [10], interleukin-8 [64], vascular endothelial growth factor [63], insulin growth factor-1 [68], and focal adhesion kinase [27]. Generally, multifactorial therapies aim to improve both the migration and trophic support provided to the ischemic tissue following stroke. In animal models, augmenting just one of these by aforementioned and other factors has consistently demonstrated enhanced functional behavior and reduction in stroke severity [66].

6.4 The Advantage and Disadvantage of Using BMSCs

Stem cells are characterized not only by their multipotent differentiation ability but also by their faculty for self-replication [12]. Hematopoietic stem cells are able to reproduce themselves while also differentiating into hematopoietic progenitor cells which are still multipotent but without the ability for self-replication [52]. HSCs

removed from bone marrow of rodents are capable of surviving and perpetuating in vitro cultures beyond the life span of the original host. However, self-replication in HSCs is not indefinite, and this phenomenon has been the focus of studies examining the effect of telomere length and telomerase activity [39]. Once removed from murine hosts, *in vitro* HSC preparations can survive up to three or four lifetimes of the original host but cannot be maintained much past this length. The inability for indefinite self-renewal may be related to telomerase activity [40].

Unlike induced pluripotent stem cells or bone marrow mesenchymal stem cells, HSCs exhibit limited capacity for expansion *in vitro*. Consequently reproducing large quantities of HSCs is challenging as they must be harvested from bone marrow itself [49], umbilical cord blood [6], or mobilized blood [7, 38]. HSCs are penchant to differentiation in cell culture rather than self-perpetuity [60]. Investigation into HSC self-renewal mechanisms *in vitro* that are absent *in vivo* is currently ongoing.

While systemic delivery of HSC via intravenous injection has proven effective in the treatment of both myocardial infarction and ischemic stroke, the mechanisms governing separate protective and restorative or regenerative effects are difficult to isolate. A key role of HSC infiltration to the ischemic brain is the anti-inflammatory effects conveyed by HSC-differentiated glia and immune cells. Tracking of transplanted BM-derived cells was traditionally conducted by Till and McCulloch by irradiating HSC from donors to induce chromosomal repairs and breaks making them distinguishable from host HSCs. In this way, fully differentiated cells with various fates could be mapped back to a single transplanted donor colony bearing the same radiation-induced chromosomal marker [4]. In contrast, recent studies examining HSC infiltration after stroke use HSCs isolated from green fluorescent protein (GFP) expressing transgenic mice that can be tracked once injected into C57/BL6 recipient mice.

Unfortunately, CD34+ cells make up approximately just 1 % of BMHSC preparations making it difficult to discern the role played by these cells. In support of the effect of CD34+ cell fraction from HSC preparations, Taguchi et al. demonstrated that cell delivery in the CD34+ group but not CD34-negative group after stroke had enhanced neurogenesis via angiogenesis. Using human CD34+ cells in an immunocompromised cell line, Taguchi et al. reported enhanced neovascularization in the peri-infarcted tissue along with enhanced neurogenesis [59].

In addition to HSCs, two other bone marrow-derived cell types have also been studied for the treatment of ischemic stroke: hematopoietic progenitor stem cells and bone marrow-derived mesenchymal stem cells. Interestingly, injection of bone marrow stem cell-conditioned media alone has been demonstrated to convey functional recovery benefits in a rodent model of stroke [11]. Thus it has been proposed that HSC transplantation can act as an augmented source for paracrine trophic support to the ischemic brain [8, 25]. This hypothesis supports the efficacy observed using HSC transplantation studies despite relatively poor homing of the transplanted cells to the brain and ischemic region itself. Despite this, the study of HSC therapy continues to seek methods for improving survival, homing, and efficacy of transplanted HSCs.

An effective approach to improving HSC transplantation therapy is the preconditioning of transplanted cells by hypoxia immediately prior to administration in an ischemic stroke model [1]. Hypoxia preconditioning (HP) has been leveraged in stem cell therapies for its multifactorial effects on improving cell survival following transplantation [28]. Both embryonic stem cells and bone marrow stem cells which undergo this hypoxia preconditioning protocol are significantly more resistant to necrotic and apoptotic insults making them better able to survive *in vitro* and *in vivo* ischemic conditions [19, 46, 61]. Preconditioning bone marrow-derived stem cells is also an effective means of improving the homing of intravenously injected stem cell preparations for the treatment of stroke and myocardial infarction [28]. Hu et al. describe a potassium channel-kinase interaction (Kv2.1-FAK)-mediated mechanism whereby hypoxia enhances the expression of Kv2.1, thereby phosphorylating FAK and boosting BMSC migration ability. Selective knockdown of the Kv2.1 channel or applying a K+ channel inhibitor significantly dampened the ability of BMSCs to migrate to the infarcted tissue. Altering gene expression in HSCs related to survival, migration, and differentiation of transplanted cells remains a popular avenue for investigation.

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Chapter 7
Endothelial Progenitor Cell Therapy in Stroke

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Abstract Endothelial progenitor cells (EPCs) are unique stem cells in circulating blood. Studies showed strong beneficial evidence using EPC therapy in experimental animal models and clinical trials. In this review, we discussed the characteristics of EPCs in the stroke therapy. We summarized the effect of EPCs on the treatment of cerebrovascular diseases including ischemic and hemorrhagic stroke, moyamoya disease, and vascular tumor, etc. Although the molecular mechanisms of EPC therapy are not fully understood, the function of EPCs included releasing growth factors, regulating microenvironment in the injury territory, and maintaining blood-brain barrier integrity. Clinical application of stem cell-based therapy is still in its infancy. The next decade of EPC research in the stroke field needs to focus on the studying the molecular mechanism or combining other type stem cells to enhance the potential of this therapeutic avenue, and translate to clinical application.

Keywords Blood-brain barrier • EPC • Ischemia • Stroke • Therapy

Abbreviations

BAVMs	Brain arteriovenous malformations
BBB	Blood-brain barrier
BM	Bone marrow
EOCs	Endothelial outgrowth cells
EPCs	Endothelial progenitor cells

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ESCs	Embryonic stem cells
GCSFs	Granulocyte colony-stimulating factors
HCC	Hepatocellular carcinoma
HGF	Hepatocyte growth factor
*h*UCB	Human umbilical cord blood
MIF	Macrophage migration inhibitory factor
MSCs	Mesenchymal stem cells
NIHSS	NIH Stroke Scale
NSCs	Neural stem cells
OGD	Oxygen glucose depletion
SMPCs	Smooth muscle progenitor cells
tPA	Tissue plasminogen activator

7.1 Stroke and Neurorepair After Stroke

Ischemic stroke is a leading cause of morbidity and mortality among cerebral disease. Irreversible injury happened both at the molecular and cellular level. These damages furthermore affect microvascular endothelium, glial cells, pericytes, and extracellular matrix, which constitute the neurovascular unit. Although collateral arteries could transiently open and supply oxygen and glucose for cell viability in the penumbra, it also trigger worsen secondary damage without an appropriate reperfusion for the timely restoration.

Neurorepair plays the most important role in the functional recovery after ischemic stroke; its target is to attenuate cerebral parenchyma injury in the acute ischemic phase and reestablish neuronal function [1]. The neurorepair processes include angiogenesis, neurogenesis, oligodendrogenesis, remodeling of neurovascular unit, and neuronal network formation. Angiogenesis involves endothelial cell (EC) proliferation, migration, microvessel sprout, capillary connection formation, and the increases of the vascular bed resistance in afflicted artery [2]. Arteriogenesis contributed to the collateral flow in the early phase of ischemic stroke, while angiogenesis improved cerebral blood perfusion in the late phase of ischemic stroke [3]. Cerebral ischemia can also induce neurogenesis in both hippocampal subgranular zone (SGZ) and sub-ventricular zone (SVZ) within the adult mammalian [4, 5]. New myelination of oligodendrocytes induced by stroke is also involved in cerebral ischemia repair [3]. Neurovascular unit, consisting of astrocytes, neurons, and vascular structures and their interactions, regulates the blood-brain barrier (BBB) and plays a critical role in physiological and pathological response; its remodeling is the basis for functional recovery after injury [6]. Thus, the longer-time recovery of neurological function regulated by all of the elements and enhancing neurorepair of the damaged tissue could be the promising therapeutic approach.

Currently, tissue plasminogen activator (tPA) is the only US FDA approved drug for patients with ischemic stroke onset within 4.5 h. Patients who have the time to

receive this treatment are only 2–5 % [7]. Although a clinical study showed that thrombolytic treatment remains effective at the first 24 h [8], its use still has limitation for the therapy of patient suffering acute ischemic stroke.

Cell-based therapy, especially stem cell based, seem attractive to neurorestoration due to their wide therapeutic window and high rehabilitation potential. Various cell types such as mesenchymal stem cells (MSCs), neural stem cells (NSCs), embryonic stem cells (ESCs), and EPCs show their efficiency in both experiment and clinical trials [8]. Although it still has practical and clinical issues like transplantation time windows, routes of cell administration, and potential detrimental effects, current studies show that stem or progenitor cells play beneficial role by paracrine and systemic effects, mostly via releasing chemokine, growth factors, and nanoparticle [9].

7.2 Current Clinical Trials of EPC Therapy in Stroke

A series of clinical trials focus on EPC in the past decade. The results showed EPC may be a characteristic biomarker in vascular diseases [10–13]. Currently, two clinical trials are ongoing using EPCs as treatment for clinical stroke patients, while no clinical trials have been published yet. Chen et al. conducted a multicenter, single-blind, randomized, parallel-controlled clinical trial (NCT01468064) on stroke patients aged 18–80 years, within 7 days of the onset of symptoms. They intravenously infused 2.5 million autologous EPCs per kg suspended in 100 ml normal saline with 5 % autologous serum approximately 4 weeks after bone marrow (BM) aspiration. Second study used the same amount of cells transplanted approximately 1 week after initial boosting. NCT02605707 is a single-blind trial studying the effects of autologous EPCs plus conventional treatment including rehabilitation on patient aged 18–80 with stroke history between 6 and 60 months and NIH Stroke Scale (NIHSS) score of 7 or more points. The outcome showed the change of a serial neurological examination after transplantation (ClinicalTrials.gov identifiers: NCT01468064 and NCT02605707).

7.3 Endothelial Progenitor Cells

EPCs were initially isolated from adult peripheral blood and capable of differentiating into an endothelial-like phenotype cells in 1997. Traditional view that ECs differentiation was occurred only in embryonic development was challenged [14]. $CD34^+$ cells derived from bone marrow can mobilize to the peripheral circulation and involve in vascular recovery [15]. Data from Takahashi Laboratory had shown that circulating EPCs mobilize endogenously as natural response foci to promote neovascular formation [16]. The level of circulating EPC is considered as a biomarker in cardiovascular and cerebral vascular disease. The reduction of EPCs

indicates the risk factor or more susceptibility to development of disease [17, 18]. The beneficial role of EPCs has been focused the valuable capability to improve the treatment of ischemic disease as a cell-based therapy.

EPCs as one of the adult stem cells originate from hematopoietic cells. It can also be found in the peripheral blood in adults. Evidence suggest that hematological stem cells (HSCs) and EPCs are derived from a common precursor [19, 20]; thus it has been speculated that circulating EPCs originate from hematopoietic stem cell [14]. Human adipose-derived stem cells can also be differentiated into EPCs via microenvironment, and the protective effect of EPC was further demonstrated in the hypoxic-ischemic injury model [21]. MSCs are capable to differentiate into cells with phenotypic and function features of endothelial cells *in vitro* [22]. Other alternative sources of EPCs include ESCs, fetal umbilical cord blood, and induced pluripotent stem (iPS) cell [23].

In in vitro cell culture, two main protocols have been used to isolate EPCs: one is primary cell culture and the other is colony assays based on surface markers. EPCs can be isolated from bone marrow, peripheral circulating blood, human umbilical cord blood (*h*UCB) [24], fetal liver, etc. Currently, the specific marker for EPC is controversial, and early or late endothelial progenitor cells have their putative markers. Pathological conditions such as cerebral ischemia, limb ischemia, and acute myocardial infarction can increase the number of EPCs rapidly in the circulation [16, 25, 26]. These EPCs were supposed to migrate from the bone marrow.

Considering the uncertain specific markers, studies use groups of markers to identify the proximate population. Asahara et al. first described EPCs as $CD34^+$/vascular endothelial growth factor receptor $(VEGFR)^+$ cells [14]. CD34 is widely recognized as the principal marker to identify EPCs, but it can also be expressed in ECs. Some researchers believed $CD133^+$ is the marker for more immature or putative EPCs [27, 28]. Human KDR and mouse Flk-1 are the receptors for VEGFR, widely expressed on mesoderm-derived lineages [29]. Mihail supposed that the loss of $CD133^+$ and a parallel expressing von Willebrand factor (vWF) may indicate EPC maturation [30]. EPC mobilization from bone marrow is regulated by a variety of factors, and it may be a part of the inflammatory response [31, 32]. EPCs can be activated by angiogenic growth factors from the periphery, especially associate with the level of endogenous VEGF [33]. EPCs then migrate to the systemic circulation, resident to the injury endothelium via the induction of cytokines and their receptors. Furthermore, EPCs participate in the cardiovascular disease and the cerebral vascular disease via promotion of angiogenesis, neovascularization, and regeneration after ischemic injury [34–36].

EPC tracking is a property approach for monitoring in vivo. MRI is an effective tool in tracking transplanted stem cells following cell labeling with superparamagnetic iron oxide (SPIO) nanoparticles [37]. Intravenously injected magnetically labeled EPCs with ferumoxide-protamine sulfate (FePro) in mice can detect tumor development by MRI [38]. *Lentivirus* packaging fluorescein is also a candidate for the track location lenti-PGK-TdTomato or GFP transduction to EPCs. Thébaud developed a qualification of fluoresce label, which has less chromosomal aberration and suitable for experiments in vivo [39]. EPCs stained with Dil-Ac-LDL also have

been used for tracking after injecting into animals [40]. Hence, these tracking technologies provide useful approaches for the EPC-based therapy.

7.4 The Function of EPCs

Generally, EPCs are generated from the BM and stay quiescently in the stem cell niche [41]. When endothelial injury such as heart attack or ischemic stroke occurs, EPCs are capable to mobilize into circulation and participate in endothelial repair and neovascularization through the differentiation into ECs and secretion of various growth factors and cytokines [42, 43]. EPCs can secrete protective cytokines and growth factors to promote the function of repair after ischemic injury [44–46]. Neurovascular niche refers to factors release after brain injury to create a supported niche and thus lead to promote post-stroke neurogenesis [5]. Stromal-derived factor 1 (SDF-1) and angiopoietin-1 (Ang-1) are upregulated after ischemic stroke that induce angiogenesis and furthermore link to neurogenesis in the unique "neurovascular niche." It is noted that neuroblast migration was abolished when the angiogenesis was inhibited. ECs also are critical components to secrete soluble factors that maintain CNS stem cell self-renewal and neurogenic potential [47].

EPCs in ischemic injury regeneration have the ability of mobilization, migration, differentiation. Several signal transduction pathways are involved in these processes and regulate the repair of injury. SDF-1/CXCR4 axis is one of signal for stem cell homing [48]. Our group demonstrated that SDF-1α promoted angiogenesis and neurogenesis during the post-acute phase of ischemia [49]. SDF-1/CXCR4 can also cooperate with VEGF/VEGFR, GCSF/stem cell factor (SCF), etc. [50, 51]. EPCs can express endothelial nitric oxide synthase (eNOS) [52], and eNOS-dependent signal influences EPC migration and correlation to insulin-like growth factor-binding protein (IGFBP-3), and estrogen receptors, modulating the function of bone marrow-derived EPCs in both autocrine and paracrine, contributing to vascular repair [53, 54]. Many factors such as soluble intercellular adhesion molecule, granulocyte-monocyte colony-stimulating factor, hepatocyte growth factor, interleukin-6 (IL-6), IL-10, estrogen, and endothelial nitric oxide synthase (eNOS) participate in these activating processes. Both tissue factors and EPC surface receptors are involved in the EPC homing process. In addition, many factors such as CXCR2 and its ligands CXCL1 and CXCL7, CCL5/CCR5 promote EPCs migration [55–57]. In a hypoxia precondition of *h*UCB, HIF-1α-to-Epac1-to-matrix metalloproteinase (MMP) signaling pathway is required for the improved therapeutic efficacy [55–58]. MMPs play a key role in the formation of vascular networks by EPCs; MMP-9 deficiency disturbed the function of EPCs and further impaired neovascularization [59]. MMP-9 is found as a novel functional cell surface marker to identify proangiogenic cells from early EPCs [60]. Hypoxia-controlled MMP-9 overexpression shows its promising approach of gene therapy for ischemic stroke during the delayed phase of ischemia [61].

To maintain normal brain activity and reaction of physiological and pathological conditions, several types of brain resident cells and long-distance cells to work together are required. Studies suggested EPC regeneration depended on their differentiation ability or a paracrine approach [14, 16, 62]. In the interaction of EPCs and ECs, an in vitro study showed that RAGE plays a positive role [63]. Considering astrocytes as an important component of BBB, astrocytes can cross talk to EPCs and increase EPC proliferation both in vitro and in vivo [64]. A combined transplantation of EPCs and MSCs suggests the cross talk happened between progenitor and stem cells [65, 66]. An in vitro study uses a coculture model that showed a higher proliferation and vascular network formation [67]. Smooth muscle cells (SMCs) also communicated to EPCs. Cocultured EPCs and SMCs stimulated abundant cytokines to release in vitro and increase capillary density and improve blood perfusion in a model of cerebral ischemia in rat [68]. It is noted that EPCs could also give a rise to smooth muscle-like progeny.

BBB disruption occurs in the acute phase of ischemic stroke. Neovascularization stimulating agent like VEGF could decrease infarct volume in the ischemic penumbra and improve outcome; however it also increased BBB permeability and resulted in brain edema. EPCs might reverse the hypoxic state by neovascularization and repair ischemia-induced endothelial injury. EPCs transplantation could improve vascular integrity, which indicating their ability in the BBB repair after injury [40]. Although EPC-based therapy may damage BBB, there are many studies showing that EPCs improved the reconstitution of BBB via angiogenesis and vasculogenesis [69].

7.5 EPC Therapy in Hypoxia and Hypoglycemia In Vitro

Generally, ECs sense hypoxia and subsequently result in redox-mediated cell activation and trigger a series of inflammation by releasing cytokines, chemokines, and growth factors. EPCs can respond to hypoxia, hypoxia-inducible factor-1α, VEGF, or erythropoietin-induced EPC activation and mobilization from the BM [69, 70].

EPC function could be regulated by various factors, which acts in a paracrine fashion leading to endogenous EC proliferation. The increase of circulating EPCs is endogenously mobilized in the BM in response to tissue ischemia or exogenously by cytokine therapy; this increase will enhance neovascularization of ischemic tissues [16]. Angiogenesis occurred in response to cerebral ischemia. Hypoxia triggers inflammatory cell infiltration and furthermore promotes local neovascularization [16, 71]. Insulin-like growth factor (IGF)-binding protein-2 (IGFBP-2)-treated human umbilical vein ECs (HUVECs) significantly enhance the incorporation of EPC tubule formation and facilitate neovascularization under oxygen glucose depletion (OGD) condition [72]. Two types of EPCs were used most in in vitro study. “Early EPCs” obtained from a short-time culture for 4–7 days are thought to derive from myeloid lineage and “late EPC” also called “outgrowth endothelial cells (OECs)” from a long-term culture for 2–4 weeks. Late EPCs showed more capable

to promote the formation of capillary-like structure when placed on Matrigel-coated dish [73].

EPC therapy in OGD model limits the progression of secondary axonal injury [74]. After a 90 min OGD treatment in primary neuron cells, the number of dead cells significantly decreases when EPCs were cocultured, while an increase in axonal degeneration appeared when subsequently cocultured with EPCs after subjected to OGD. The development of neurons is guided by molecules, which are provided by blood vessels. Some molecules can be the inductor to guide both neovasculature and neurogenesis. The interdependence between vasculatures and neurons suggested that EPCs are an important mediator of axon survival and vascular protection. The therapeutic effects on axons showed that EPCs played its role by releasing paracrine trophic factor. $CD34^+$ cells provided molecules such as VEGF, FGF-2, and IGF-1 and showed to accelerate endogenous neurogenesis [75–77]. EPCs had both vaso- and neuroprotective potential after OGD on hippocampal organotypic slices (OHC), which suggested paracrine mechanism of EPC-dependent protection [78]. The release of TGF-ß1 and IL-6 is slightly enhanced accompanied by the increase of TLR-3/4 ligands.

In vitro studies of EPCs combing growth factors or cytokines may enhance EPCs function [79]. For example, as chemoattractants, both SDF-1 and VEGF increased EPC migration; a combination of VEGF and SDF-1 caused an additive increase in EPC migration. SDF-1, VEGF, and FGF-2 could promote the formation of tubelike structures in Matrigel by testing tubelike formation of EPCs. Hypoxia upregulated chemokine receptors CXCR2 and CXCR4 in EPCs and triggered SDF-1, VEGF, CXCL1, and macrophage migration inhibitory factor (MIF) release. Consequently stimulating EPCs enhanced tube formation [80]. Gene transduction indicated that hepatocyte growth factor (HGF) and microRNAs promoted a much greater tubelike structure in ischemia, which suggested microRNA and related proteins are involved in EPC-based treatment [81].

7.6 EPC Therapy in Ischemic Brain In Vivo

Cell-based therapy has been used as a strategy to promote neovascularization and regeneration after ischemia injury. Delivery of culture-expanded EPCs for transplantation required property approaches; three different strategies have been principally used. The first strategy is systemic administration or intravenous delivery of EPCs, which is injected usually from tail vein, femoral vein, or jugular vein. This strategy improved neo-vascularization in the cerebral ischemic zone and functional recovery after middle cerebral artery occlusion (MCAO) [82]. The second strategy is intra-arterial delivery; studies showed that mice got a better outcome after the injection of ex vivo-expanded autologous BM-derived EPCs by internal carotid artery (ICA) [54]. Since intra-arterial infusion could cause embolism, intravenous infusion is believed to be the optimal route, which is proper for the clinical application [83]. The third strategy is striatal delivery, which means cells are directly

injected to the striatal area, or the ischemic core. Administration directly to the injury core is assumed to be more effective. Therefore, both routes of cell administration were assessed for producing the greater recovery. The result showed that intravenous delivery may be more effective than striatal delivery in producing long-term functional benefits to the stroked animal [84, 85]. Intracerebral CD34+ cells transplantation could enhance endogenous stem cell mobilization, homing and engraftment into the brain [86]. Furthermore, a fourth approach, nasal administration, was also mentioned in cell-based brain ischemia therapy. Intranasally administered cells could penetrate the BBB into the brain parenchyma and cerebrospinal fluid (CSF) [87]. Intranasal delivery of MSCs improved functional outcome in a model of ischemic brain injury, indicating the efficiency of the nasal route [88]. As for the dose, the range of $0.2–3.0 \times 10^4$ EPCs per gram body weight shows satisfactory efficacy in various ischemic animal models [82, 89–91]. For human application in the future, the dose of EPC and the administration time window shall be carefully considered. The clinical trial NCT01468064 is designed to intravenously administrate 2.5×10^6 EPCs per kilogram body weight. They transplanted EPC at 4 weeks after BM aspiration, and second transplantation was implemented at 1 week after initial boosting. Another clinical trial NCT02605707 is designed to use autologous EPC transplantation for the chronic ischemic stroke, but it did not show the detailed information.

EPCs have the stem/progenitor cell and hematopoietic characteristics and could be stimulated by ischemia circumstance. Once EPCs are recruited to the injured tissue, it undergoes endothelial regeneration and eventually has a better outcome. Circulating BM-derived EPCs contribute to neovascularization and microvascular structure after MCAO in the adult brain [34]. Circulating human EPCs showed homing to the ischemic brain in 24 h after delivery, therefore EPCs play beneficial role both at delayed and acute phase [82]. Autologous peripheral blood-derived EPCs have a minimal risk to the host during EPC isolation and required minimal manipulation before transplantation [92]. Autologous transfusion of peripheral blood-derived EPCs via the internal carotid artery had been shown to reduce the brain infarct zone (BIZ) and neurological deficit in a rat model of MCAO [93]. Transplantation of *h*UCB-derived EPC promotes functional recovery through the magnitude of endogenous proliferation, angiogenesis, and neurogenesis; considering the controversial marker to definite EPCs, some study use *h*UCB $CD34^+$ and $AC133^+$ cells [94, 95]. These kinds of EPCs may also serve as a property agent for homologous or autologous transplantation in ischemic tissue.

Considering SDF-1/CXCR4 signaling played an important role in the activation of EPCs, overexpression of SDF-1/CXCR4 genes showed better outcomes compared to the controls. Hiasa demonstrated that SDF-1α gene transfer enhanced recruitment of EPCs and promoted vasculogenesis in vivo [96]. The effectiveness of SDF-1α gene transfer into a mouse MCAO model suggested that SDF-1α facilitated to recruit progenitor cells in the brain and consequently benefit stroke recovery. AAV-SDF-1α expression represents a promising avenue for ischemic stroke therapy [49]. Furthermore, insulin or the combination of SDF-1α and VEGF increases EPC-mediated neovascularization [97, 98]. microRNAs are small, noncoding, single-

stranded RNAs that play a key role in regulating EPC function [99]. For example, miR-126, miR-130α, and miR-221/miR-222 regulate the proliferation of EPC; miR-21, miR-34a, and miR-22 regulated senescence of EPC; miR-126 and miR-150 regulate cell migration of EPC; and miRNA-107, miRNA-16, miRNA-34a, and miRNA-126 are related to EPC differentiation [1, 100–107].

Several studies have shown that the administration of early EPCs is associated with a better outcome after ischemic injury. Endothelial outgrowth cells (EOCs) also known as endothelial colony-forming cell populations of human EPCs are particularly promising for vascular tissue engineering applications [108]. OECs are also known as late outgrowth or endothelial colony-forming cells (ECFCs). Although there is no single specific marker for this cell population, OECs could be obtained under cell culture and are capable to form vessel-like structures [109, 110]. OECs isolated from *h*UCB could improve functional recovery by intravenous injection to MCAO rat after 1 h reperfusion [89].

Moyamoya disease (MMD) is a cerebral vascular disease in which certain arteries are constricted and thus creates a chronic ischemic circumstances. Abnormal neovasculature in moyamoya undergo vasculogenesis, and the differentiated mature ECs go through a pathological way [111]. It was reported children with MMD had abnormal cluster formation [112]. The number of EPCs was significantly reduced with less tube formation and increased senescent-like phenotype. MMD patients had a significant increase in circulating EPC mobilization. Circulating EPC number reflected mixed conditions of abnormal vasculogenesis, while OEC number was frequently detected in moyamoya vessels [113]. As circulating EPCs were closely involved in the initiation and development of moyamoya disease, it was possible to manipulate EPC behaviors to lead to a better outcome [114].

7.7 EPC Therapy in Other Cerebrovascular Diseases

In order to prevent secondary neuronal injury in intracerebral hemorrhage, vascular repair could be a potential target for the nerve regeneration [115]. Neurological deficit was diminished when EPCs were intravenously administrated on the first day after intracerebral hemorrhage in rats [116]. One of limitations of EPC therapy was the number of EPC injection. Therefore, searching for molecules to regulate EPC function may be essential targets in the ICH therapy. Furthermore, SDF-1α was always considered to play a key role in the recruitment of EPCs and supported revascularization in ischemic tissue. In a rodent model of ICH, delivering SDF-1α together with EPC resulted in significantly increased blood vessel formation [117]. In a hemorrhagic stroke model, EPO pretreatment promoted EPC mobilization and therefore significantly attenuated tissue injury and dysfunction [118]. The level of late EPCs could serve as a biomarker in patients with hemorrhagic stroke [119]. It is noted that EPCs had a therapy potential in aneurysm, which promoted endothelialization of the coiled aneurysm neck via induction of EPCs [120].

Brain arteriovenous malformations (BAVMs) were congenital vascular lesion that resulted in deficiency to form the capillary bed and involved the vasculogenesis of blood vessels and could cause intracranial hemorrhage [121]. Since there are a higher number of EPCs and SDF-1 expression in BAVM, SDF-1 in EPC-treated BAVM may promote vasculogenesis and inflammatory cell migration [122]. The number of EPCs and the expression of SDF-1 were increased in BAVM patients. EPCs are activated by SDF-1/CXCR4 signaling, which are recruited to form abnormal vasculature cluster in BAVM patients. Furthermore, EPCs may mediate pathological vascular remodeling and affect the clinical development of AVMs due to its presence in the nidus of the brain and spinal cord AVMs [123]. The higher-staged BAVMs increased attractive factors and then recruit EPCs [124]. The number of proliferating ECs changed in stage III and recruited EPCs and finally resulted in significantly increased abnormal vasculature cluster. Further evidence is warranted to determine whether EPCs are a suitable potential agent for the BAVM to turn to normal structure.

EPCs could mobilize in response to hypoxia condition, then promote vasculogenesis, and result in vascular generation in the pathological tissue [125, 126]. During tumor development, SDF-1/CXCR4 and VEGF/VEGFR pathways are known as mediators of EPC mobilization. Therefore, potential target may focus on new anti-vasculogenic approach [127]. Characterization of tumor-associated EPCs in hematological tumor provides the possibility of specific anti-vasculogenic therapy [128]. EPCs mediated the “angiogenic switch” and induced cancer cell metastases, which suggested EPCs represent a therapeutic target both in early and late stage of cancer progression [129]. A study showed circulating EPCs are able to predict an increased risk of non-small cell lung (NSCL) carcinoma recurrence and death in an early post-surgery [130]. BM-EPCs were reported close to hepatocellular carcinoma (HCC). Injection of EPCs specifically homes to tumor tissue and plays a prominent role in HCC neovascularization. Blockade of BM-EPC-mediated vasculogenesis improved the therapeutic efficacy for the HCC [131]. EPCs could be used as a candidate prognostic and predictive biomarker for gastric cancer [132]. Systemic delivery EPCs transduce with an immune-activating gene could alter the tumor immune microenvironment and lead to a therapeutic effect of murine ovarian cancer [133]. Thus, therapy aimed at EPCs should be taken into consideration for cell-based therapy for tumor or as a potential vehicle for gene or molecule therapy. Studies also established to use labeled EPCs to investigate the migration and incorporation of EPCs into tumor neovasculature [134].

7.8 Combination Therapy to Improve EPC Efficacy

Although EPC-based therapy attracts attention on experiment and clinical trials, results were not satisfactory enough. Tumorigenicity and limited resources confined application of EPCs. Strategies to enhance the efficiency of EPCs are combination therapy including co-transplantation of different types of cells, adjunct treatment, or

ex vivo modification to improve EPC function by molecule factors. Using genomic and proteomic combination approach showed that TNF-α could increase EPC migration and incorporation into vessel-like structures [135]. HMGB1 upregulation could promote exogenous *h*PB-EPC-mediated stroke recovery by modulating paracrine function of *h*PB-EPCs in a model of MCAO [136]. Osteopontin (OPN) played a role in the homing and incorporation of EPCs to the site of endothelial injury [137]. Endothelial nitric oxide synthase (eNOS)-mediated angioprotective function and the combination of eNOS transfection and OPN produced more focal adhesions and spreading area. Combination of autologous EPCs and OPN coatings could be a promising method of developing functional endothelialized surfaces [138, 139]. The combinatorial granulocyte colony-stimulating factors (GCSFs) and EPCs were also proven to produce synergistic effects in ischemic stroke [140]. In diabetic ischemic stroke model, EPCs could be accelerated to senescence and apoptosis mainly due to p38 mitogen-activated protein kinase (MAPK) activation. Bai showed the synergistic efficiency of combination of EPC transplantation and a p38 inhibitor RWJ 67657 administration. Suppression of p38 signaling increased the number of EPCs and improved neurological function and thus accelerated recovery from diabetic stroke via promotion of angiogenesis and neurogenesis [141]. Erythropoietin (EPO) promoted post-stroke angiogenesis by stimulating endogenous EPC proliferation and migration [142]. Furthermore, EPO administration in combination with EOCs showed synergistic effects on neurological recovery, angiogenesis, and neurogenesis in rat MCAO model [52]. Study also showed that EPCs possibly favor the proliferation of mononuclear cells (MNCs) and HSCs. The concentrations of IL-6 and VEGF were upregulated and might be related to hematopoietic reconstitution and homing ability [143].

As mentioned above, EPCs have a late population, called OECs. OECs have different functions compared to EPCs; the combination of EPCs and OECs showed synergism during neovascularization. These two cell types augmented the angiogenic capability in vitro and showed better outcome compared to any single-cell-type transplantation [144]. Neuroblast survival was needed to mature vascular network [145], as smooth muscle cells/pericytes were needed for the maintenance of BBB integrity during cerebral ischemia [146]. The intravenous coadministration of *h*UCB-derived smooth muscle progenitor cells (SMPCs) and EPCs in mice got better outcomes than administration of one single type alone. The results showed that more mature vascular remodeling, more cell proliferation, and neuroblast migration occurred in both peri-infarct and infarct area [145]. Transplantation of NSCs or neural precursor cells (NPCs) is also used to repair the nerve system because of the poor self-regeneration ability in CNS [147]. Adipose-derived stem cells differentiated toward EPCs and NPCs; and the combination of both cells produced significant reduction of hypoxic-ischemic brain injury [21]. It is possible that NPCs and EPCs have cell-cell interaction during cell migration; and hypoxic microenvironment could enhance cell-cell connection. A clinical trial in refractory ischemia used the combination infusion of MSCs and EPCs which showed significant improvement in quality of life according to the phase I [148]. The improved outcome suggested that this therapy is safe and feasible.

Besides induced by molecule and combination with various cells in EPC-based therapy, magnetic guide also used to enhance therapeutic effect. Labeled EPC with SiO_4@SPIONs showed the efficient guide by exterior magnetic field toward ischemic hemisphere [149].

7.9 Mechanism of EPC Therapy in Stroke

EPCs possessed the capability of differentiation and neovascularization. Studies provided evidence that neurological benefits coupled with angiogenesis [89]. After ischemic stroke, EPCs were stimulated and recruited to the ischemia brain and promoted recovery. When ischemic injury happen, SDF-1α was highly increased, which resulted in recruitment of CXCR4 that generally express in EPCs. Therefore, EPCs could perform their functions to promote the repair of injured brain. SDF-1/CXCR4 axis also cooperated with growth factors such as VEGF/VEGFR, KDR/CD34, and GCSF/stem cell factor (SCF) [13, 50, 51]. SDF-1α/CXCR4 axis possessed capability triggering cell proliferation and anti-apoptosis signals such as MAPKs and PI3K and the serine/threonine kinase Akt [150]. High CXCR4-expressed EPCs increased capillary density in ischemic hind limbs [151].

Endogenous nitric oxide (eNOS), increased following ischemic stroke, is one of the factors promoting EPC mobilization [54]. Chen demonstrated that eNOS was a downstream mediator for VEGF release and angiogenesis. Furthermore, eNOS also regulated BNDF expression and neuronal progenitor cell proliferation, migration, and neurite outgrowth, finally affecting function recovery after cerebral ischemia [152].

HIF-1α is a transcription factor that regulates the adaptive response to hypoxia in cells. HIF-1α expression upregulated VEGF and EPO levels in hypoxia brain [134]. VEGF/VEGFR signaling further leads to the growth of new vessels after ischemic stroke [153].

Early EPCs secreted abundant proangiogenic cytokines including VEGF, transforming growth factor-β (TGF-β), macrophage colony-stimulating factor (MCSF), placental growth factor (PGF), HGF, fibroblast growth factor (FGF), MIF, thrombopoietin (TPO), and interleukin-8 (IL-8). It could also secrete a few anti-angiogenic cytokines and neurotrophic and neuroregulatory cytokines including brain-derived neurotrophic factor (BNDF) [154, 155]. EPC mobilization and recruitment to ischemic area could be a very complicated process. Identification of these regulators and understanding of their function provided clear knowledge of EPC-based therapy.

EPCs are involved in growth factor secretion, cell proliferation, neovascularization, and neurovascular unit repair, which suggested that EPCs played an important role under certain conditions such as tumor progression, myocardial infarction, and stroke [14, 15, 34, 156, 157]. However, circulating EPCs promoted the vascular repair or angiogenesis mainly by releasing paracrine factors such as VEGF or HGF to activate resident ECs rather than self-proliferation from the myeloid-monocytic lineage. Resident ECs primarily contributed to endothelial regeneration [158]. EPC-

induced angiogenesis may include a large quantity of ECs by proliferation and differentiation or an increased supply of growth factors to activate resident mature ECs [159]. Therefore, the secretion of angiogenic growth factors such as VEGF, HGF, and granulocyte-macrophage colony-stimulating factors (GM-CSFs) from EPCs contributed to the proangiogenic effect. That means circulating cells may involve the regulation of vascular repair via paracrine mechanisms. In a model of MCAO-based diabetic animals, monocyte function could be reduced and the neovascularization is attenuated [160].

Neurorepair required the replacement of dead neurons and reconnection of neuronal network. Ischemia triggered the activation of several endogenous progenitor cells such as NPCs, EPCs, or oligodendrocyte progenitor cells (OPCs). Neurogenesis in the adult brain occurred in two areas: the hippocampal subgranular zone (SGZ) and sub-ventricular zone (SVZ) [4, 5]. Hypoxia was a strong activator of neurogenesis in CNS, since hypoxia-inducible VEGF and its receptor VEGFR-2/Flk are expressed in NSCs in vitro. VEGF was supposed to acts as trophic factor for NSCs in vitro and for sustained neurogenesis in the adult CNS [161]. Human NSCs expressed CXCR4 and migrated toward the infarct area, where SDF-1α is upregulated [161, 162]. Thus secretive factors of EPCs such as VEGF and SDF-1 provide a suitable microenvironment for neuronal regeneration and survival. Evidence showed that progenitor cells like NPCs were capable to differentiate to matured neurons and glia; the angiogenic environment was required to promote effective neurogenesis and survival [75]. Neurons could be formed in the SVZ and stimulated to migrate toward the ischemic boundary region [163]. Neuronal migration is influenced by cell-secreted factors and by cell-bound molecules including gamma-aminobutyric acid (GABA), VEGF, BDNF, polysialylated neural cell adhesion molecule (PSA-NCAM), matrix metalloproteinases (MMPs), β1-integrins, angiopoietin (Ang), and extracellular matrix components [164]. Angiogenesis, neurogenesis, and synaptic plasticity were endogenous processes, which could be activated under pathological conditions in adult brain. Administration of $CD34^+$ cells enhanced angiogenesis and increased neuroblast migration from SVZ to the ischemic region, which suggested that a stable neovasculature was essential to support neuronal regeneration [91]. Palmer provided for the first time that adult neurogenesis occurred within an angiogenic niche [165]. Signals that recruit neurogenesis are known as angiogenic factors such as FGF-2, and some factors responded to angiogenic factors including FGF, TGF-α and TGF-β, and PDGF [166–168]. These may modulate both angiogenesis and neural regeneration within a "vascular niche."

Recently, novel modes of neurovascular regulation were being highlighted, such as the microRNA (miRNA) modulation and microparticle or microvesicle release. miRNAs are small, mainly 20–24-nucleotide, noncoding, single-stranded RNAs with gene regulatory activities [169]. miRNAs regulated EPC functions including EPC proliferation, mobilization, migration, differentiation, and tube formation [99]. miRNA-221 and miRNA-222 are transcribed from the same miRNA cluster and showed to regulate EPC proliferation. It has been reported that overexpression of miR-221 in EPCs could attenuate EPC proliferation [170]. It is demonstrated that

the level of miR-221 had a correlation to the number of EPCs in vascular disease like cardiovascular disease and atherosclerosis [107].

MiR-126 as one of the most abundant miRNAs in ECs governs vascular integrity and angiogenesis [171]. miR-126 regulated VEGF-dependent PI3K and MAPK signal pathway, known as the negative regulators of VEGF. Inhibition of VEGF signal resulted in defects similar to the miR-126 knockdown in a zebrafish, which indicated that miR-126 could be a target for modulating vascular integrity and angiogenesis [172]. Further study demonstrated that miR-126 could have an ability to promote HUCBC-induced neuro-restorative effect after ischemic stroke in type 2 diabetes mellitus rodents. miR-126 expression was increased and coupled with M2 macrophage polarization after HUBCB treatment in a stroke model of type 2 diabetes mellitus rodents. These data supported that the functional recovery attributed to miR-126 mainly resulted from the neuro-restorative rather than neuroprotection [173].

Other miRNAs are also involved in EPC function. Knowing that CXCR4 is a target of miR-150, Rolland-Turner demonstrated that miR-150 is involved in EPC recruitment to the ischemic heart under ischemic condition [174, 175]. miR-31-miR-720 pathway is shown critical to EPC activation and regulated EPC-induced angiogenesis after brain ischemia [176]. miR-31-5p induces EPC migration/invasion by targeting FAT4 and promoted angiogenesis [177].

Microvesicles (MVs) have small particle size, 0.1–1 μm, carrying proteins and mRNAs, miRNAs, DNAs, and cytokines and serving as veritable vectors for intercellular exchange of information, which could be shed from various cell types [178]. MVs released from EPCs had the capability of triggering angiogenesis via a horizontal transfer of mRNAs or miRNAs [179]. MVs from serum-free EPCs medium showed beneficial effects on hypoxia/reoxygenation human brain, which may be due to carry their parent proteins and genetic materials [180]. EPC-derived MVs incorporated in ECs could promote EC survival and proliferation and form capillary-like structures by a horizontal transfer of mRNAs [179]. These findings indicated that EPC-MVs could not only be a potential mechanism of EPC-based therapy, but also provide a novel vehicle for cerebral ischemia therapy.

7.10 Conclusions

During recent years, numerous studies demonstrated that EPCs played an important part in cerebrovascular disease including ischemic stroke, moyamoya disease, hemorrhagic stroke, vascular tumor, etc. EPCs showed as a promising tool in cerebrovascular therapy. Stem cell-based therapy in ischemic stroke is still in its infancy. EPC-based therapy in ischemic stroke has been highlighted not only because of its capability to establish connection in angiogenesis, neurogenesis, oligodendrogenesis, and whole neurorepair niche but also of its involvement in BBB repair and functional recovery. Although the pivotal role was investigated in ischemic response and therapy potential both in vitro and in vivo, the in-depth mechanisms are still

needed to be discovered. As rapid technological advances and novel modes of molecule regulator is being uncovered such as paracrine factors, miRNAs, and microvesicles, the strategies of combination therapy to optimize the function of EPC-based therapy could be the promising application. Further studies should be focused on the definitive markers of different types of EPCs, mechanisms pertaining to function of EPCs, and standardization of EPC administration in ischemic stroke. Challenges remain when facing the translation from in vitro or in vivo animal studies to clinical trials. Preclinical studies and clinical research were needed to improve the benefit and minimize risks as well.

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Chapter 8
Co-transplantation Strategies and Combination Therapies for Stroke

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Abstract Worldwide cerebrovascular disease (CVD) is increasing in parallel with modernization, changes in lifestyle, and the growing elderly population. The incidence of stroke increases significantly with age both in men and women with incidence rates accelerating above 70 years. Since stroke afflicts mostly the elderly comorbid patients, it is highly desirable to test the efficacy of cell therapies in an appropriate animal stroke model. All monotherapeutic attempts to prevent or ameliorate brain damage following stroke have failed so far. In view of previous findings indicating that stroke impacts a wide range of mechanisms, ranging from central nervous system (CNS) physiology over CNS regeneration and plasticity to the adaptive immune system in an age-dependent manner, the failure of monotherapies is perhaps not unlikely. Bone marrow-derived mesenchymal stem cells (BM MSCs) and hematopoietic stem/progenitor cells (HSPC) are the most frequently cells used in preclinical and clinical neurorestorative studies in stroke therapy. Therefore co-transplantation of BM MSCs with other cells may be a better strategy to improve microenvironment, make the grafting more efficient, and improve functional recovery after stroke. Current knowledge includes: (1) the potential for neurogenesis is also preserved in aged, stroke-injured brains; (2) the environment of the aged brain is not hostile to transplantation of BM MSC; and (3) the extent of recovery is successful in some but not all behavioral tests. However, there remain significant developmental and translational issues to be resolved in

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future studies such as: (1) Understanding the differentiation into specific phenotypes. Upon transplantation, the differentiated cells often de-differentiate (Kalladka and Muir Stem Cells Cloning 7:31–44, 2014). (2) Tumorigenesis remains a significant concern (Riess et al. J Neurotrauma 24(1):216–225, 2007). (3) Anti-neuroinflammatory therapies are a potential target to promote regeneration and repair in diverse injury and neurodegenerative conditions by stem cell therapy. (iv) Efficacy of cell therapy can be enhanced by physical rehabilitation (Dunnett Clin Neurol 110:43–59, 2013). We recommend that in a real clinical practice involving older poststroke patients, successful regenerative therapies would have to be carried out for a much longer time. The BM MSC therapy in aged rodents warrants further investigation including repeated administrations of therapeutic cells at several time points after stroke and using various combinations with G-CSF or other relevant growth factors/cytokines.

Finally, a better understanding of potential risks of stem cell therapies in strokes shall makes the translation of cell therapies safer. Likewise, awareness of their biology may help improve their efficacy to achieve therapeutic success.

Keywords Stroke • Strategy • Combination therapies • Co-transplantation

8.1 Introduction

Cerebrovascular diseases (CVDs) represent a leading cause of death worldwide. Worldwide CVD is increasing in parallel with modernization, changes in lifestyle, and the growing elderly population. Stroke not only has a very high mortality rate but also results in debilitating neurological impairments or permanent disability in survivors associated with huge economic losses. Etiologically, ischemic strokes represent approximately 80 % of all cerebrovascular infarctions, and hemorrhagic strokes account for the remaining 20 % [51].

In demographically developed countries, the average age at which stroke occurs is around 73 years reflecting the older age structure of these countries. The probability of a first stroke or first transitory ischemic attack is around 1.6 per 1000 and 0.42 per 1000, respectively. In less developed regions, the average age of stroke will be younger due to the different population age structure resulting from higher mortality rates and competing causes of death.

Stroke patients are at highest risk of death in the first weeks after the event, and between 20 % and 50 % die within the first month depending on type, severity, age, comorbidity, and effectiveness of treatment of complications. Patients who survive may be left with no disability or with mild, moderate, or severe disability. Considerable spontaneous recovery occurs up to about 6 months [22]. However, patients with a history of stroke are at risk of a subsequent event of around 10 % in the first year and 5 % per year thereafter [31].

The negative consequences of stroke extend well beyond the victims themselves, ultimately including families, caregivers, social networks, and employers. The proportion of patients achieving independence in self-care by 1 year after a stroke ranges from around 60–83 %. This wide variation relates to whether the studies are community based or hospital based, in which activities are considered in estimating independence and the methods used to rate ability. In established marked economies (EMEs), depending on the organization of hospital services, between 10 and 15 % of survivors are resident in an institution at 1 year [5].

8.1.1 Age Is the Principal Risk Factor for Stroke

The incidence of stroke increases significantly with age both in men and women with incidence rates accelerating above 70 years [155]. However, there are age-related gender differences in the incidence of stroke. Men aged up to 75 years old are more likely to be hit by stroke than women. The risk to have a stroke then becomes higher in women than men aged 85 years or older [155]. This may be attributed to sex-related differences in the life expectancy of women and the development of age-related comorbidities. Effects of age and gender on stroke incidence, functional recovery, and mortality have not only been shown in humans but also in animal models [16, 64]. Indeed, the age-dependent increase in the evolution of ischemic tissue into infarction strongly suggests that age is a biological marker for the variability in tissue outcome in acute human stroke [7].

It should be noted that the age-associated decline in functional brain reserve is most pronounced after the age of 85 and implies an impaired response to stressors and illnesses [61]. Importantly, age-associated changes show great variability among individuals, which are modified by genetic and long-term lifestyle factors [175, 192].

8.1.2 Elderly, Comorbidities, and Stroke

Our knowledge about the molecular and cellular mechanisms underlying accelerated infarct progression in subjects with metabolic syndrome is still poor. Some studies report a strong connection between nutrition and body weight, on one hand, and increased oxidative stress or pro-inflammatory changes in the brain, which promotes neural imbalance and glucose level elevation, on the other hand [32]. Zhang and colleagues suggested that metabolic inflammatory changes in the brain are linked to the IKK/NF-kB signaling pathway [199].

Observational studies have shown a strong correlation between blood lipid levels and stroke [80]. In animal models it could be shown that VEGF-induced angiogenesis is compromised by hyperlipidemia and provided an explanation of poor efficacy of angiogenic therapies in patients suffering from hyperlipidemia [196].

Overnutrition and hypercholesterolemia may not only be responsible for metabolic inflammation of the brain but can also induce mitochondrial dysfunction and increased oxidative stress (NADPH oxidase dependent), which may promote metabolic syndrome and related diseases [32, 70]. Cellular stress and hyperglycemia are known to accelerate the aging process. In this light, a better understanding of molecular factors and signaling pathways underlying the metabolic syndrome as well as the contribution of comorbidities to stroke-induced sequelae may be translated into more successful treatments or prevention therapies against age-associated diseases which, in turn, would improve lifespan and quality of life.

8.1.3 Stroke Models Using Aged Animals Are Clinically More Relevant

Since stroke afflicts mostly the elderly comorbid patients, it is highly desirable to test the efficacy of cell therapies in an appropriate animal stroke model. Animal models of stroke often ignore age and comorbidities frequently associated with senescence, and this could be one of the explanations for unsuccessful bench-to-bedside translation of neuroprotective strategies. Worldwide stroke is increasing in parallel with modernization, changes in lifestyle, and the growing elderly population. In particular, rates in Eastern Europe have been increasing, such that currently the highest rates are found in countries such as Bulgaria, Romania, and Hungary. Among the women and men, individuals with a low-risk lifestyle (exercising daily, consuming a prudent diet including moderate alcohol and having a healthy weight during midlife) had a significantly lower risk of stroke than individuals without a low-risk lifestyle. Therefore the relatively high incidence of stroke may be due in part to the impact of numerous known risk factors in [51] arterial hypertension, diabetes, high cholesterol, smoking, alcoholism, obesity, stress, and a sedentary lifestyle.

Comorbidities like diabetes, arterial hypertension, or comorbidity factors such as hypercholesterolemia are common in elderly persons and are associated with higher risk of stroke [65]. Moreover, simultaneous presence of vascular diabetic complications and associated comorbidities like hypertension and chronic diabetes significantly increased the level of ischemic damage [152].

Currently, there are several different rodent models with comorbidities such as the spontaneously hypertensive rat (SHR) model, stroke-prone spontaneously hypertensive rat (SHRSP), the streptozotocin rat model for diabetes, and the high-fat diet or high-sugar diet Sprague Dawley rats. Diffusion (DWI)- and perfusion (PWI)-weighted magnetic resonance imaging of the ischemic penumbra in SHRSP showed increased infarct size early after stroke as compared to the normotensive WKY rats. Moreover, the infarct volume after 60 min MCAO was greater in SHRSP (36 ± 4 % of hemisphere volume) than in SHR (19 ± 5 %) or WKY normotensive rats (5 ± 2 %) [116].

High blood pressure is a major risk factor for stroke. Large clinical trials have shown that ACE inhibitors reduce the incidence of stroke by up to 43 % [195]. However, because normotensive patients also benefit from ACE inhibition, it has been suggested that these effects may also be independent of the blood pressure-lowering effects of ACE inhibition [171]. Indeed, neither short (7 days) nor long term (42 days) prior to stroke administration of ACE inhibitors to SHR reduced the infarct size despite lowering the blood pressure, while WKY normotensive rats showed, paradoxically, marked reductions in infarct volume [142].

Over the past 10 years, a variety of models of middle cerebral artery occlusion (MCAO) have been established in rodents [8]. MCAO in aged rodents has been produced with permanent or transient occlusion for 30–120 min using (1) MCA ligation after craniectomy [189] and (2) intraluminal thread occlusion [174], a hook attached to a micromanipulator [140], cauterization [50, 87], photothrombosis [200], and endothelin injection [172, 183], injection of a thrombus via ECA [50], or intraluminal thrombus formation by thrombin injection (using occlusion of distal branches of the middle cerebral artery (MCA)).

Since focal cerebral ischemia is technically difficult to perform in very old rats and since based on epidemiological studies human stroke occurs more often in late middle-aged (60–70 years old) subjects, it is advisable to use middle-aged instead of very old animals for stroke research [137].

8.1.4 Spontaneous Stroke Recovery in Aged Patients and Animals

Stroke patients regain some of their lost neurological functions during the first weeks or months after the stroke. In contrast, in animal models of stroke, complete spontaneous recovery may occur in young rats, depending on the size and location of the ischemic lesion.

In clinical practice, physical therapy is widely used for stimulating poststroke recovery [69, 75, 100] which is thought to occur via recruitment of adjacent cortical regions [67].

In animal models of stroke, young rats begin to show improvements of neurological function starting by day 2 poststroke, whereas in aged rats, neurological recovery is hardly detectable before days 4–5, with about 75 % of the functional improvement observed in young rats [28].

Housing experimental animals in an enriched environment enhances the recovery from brain damage both in young and aged animals [28]. When aged rats were allowed to recover in an enriched environment, the delay period was shortened, and behavioral performance was significantly improved. The improvement in task performance positively correlated with slower infarct development, fewer proliferating astrocytes, and smaller size of the glial scar [28]. Even more effective rehabilitation of the contralateral forelimb could be achieved by Corbett and colleagues by combining enriched environment with training [72].

8.1.5 Bone Marrow-Derived Cells and the Therapy of Subcortical Stroke

Despite improving knowledge about stroke pathology, therapeutic benefits for stroke patients are limited. However, spontaneous recovery is common, whenever the infarct is located in the striatum, a subcortical structure that exhibits activity-dependent plasticity related to movement and motor learning. This may explain why patients with subcortical lacunar stroke are more likely to have early functional recovery after stroke [15, 158]. Other studies suggest that the beneficial effect could be due to in situ secretion of neuroprotective factors by the transplanted cells. For example, human-derived inducible pluripotent cells (iPSC) implanted into the striatum of young animals at 1 week after MCAO protected substantia nigra from atrophy, probably through a trophic effect [134]. Neurological recovery in patients with subcortical stroke is associated with neuroplasticity in the contralesional striatum [148] and axonal plasticity in contralesional motor cortex [150], which may explain why patients with subcortical stroke are likely to exhibit functional neurological recovery [15, 158].

However, whether endogenous neurogenesis in the perilesional subventricular zone contributes to spontaneous recovery after stroke has not yet been established. In addition, age, comorbidities, physical condition of the patient, and severity of disease could substantially influence these steps and, therefore, the outcome of the healing process.

Several studies on aging have established that the neurogenic subventricular zone become disorganized with increasing age. During aging, ependymal cells accumulate dense bodies, become flattened and gradually lose their cilia. Both ependymal cells and astrocytes accumulate dense bodies and intermediate filaments in their cytoplasm. The number of newly born neurons within the SVZ decreases over time, while the generation of oligodendroglial cells seems to be preserved in the aged brain [34]. Similarly, the number of DCX-positive cells in the RMS and the OB from fetal to adult stages decreases with age, while the number of the newly generated non-neuronal cells, such as oligodendrocytes, seems to remain relatively constant [17, 160, 188].

8.1.6 Cell Therapy of Cortical Stroke Using Mesenchymal Stem Cells in Aged Animal Models

Due to the ethical concerns and limited availability of using pluripotent embryonic stem cells (ES) and induced pluripotent cells (iPS) in the clinic, great interest has developed in mesenchymal stem cells (MSCs), which are free of both ethical concerns and teratoma formation.

Recent studies suggest that modified mesenchymal stem cells (MSCs) are able to form a “biobridge” between neurogenic subventricular zone (SVZ) and the isch-

emic cortex area (penumbra). Using this road, endogenous stem cells can migrate from the neurogenic area to the site of lesion and may ameliorate outcome in experimental models of cerebral ischemia [54, 75, 90, 178]. Several studies also showed that grafting of BM MSCs in the peripheral circulation improved functional neurological outcome and reduced infarct volume [75].

Mesenchymal stem cells (MSCs) and hematopoietic stem/progenitor cells (HSPC) that are most frequently used in preclinical and clinical neurorestorative studies in stroke therapy augment this endogenous response. MSC can be obtained either from the bone marrow or adipose tissue [129]. HSPC can be isolated from bone marrow or from umbilical cord blood (UCB) or can be mobilized into the blood by the administration of G-CSF. Amniotic fluid has been investigated as a new cell source for mesenchymal stem cells in the development of cell-based transplantation. Earlier studies have demonstrated the ability of amniotic fluid-derived stem cells to differentiate along a neurogenic pathway [44].

8.1.7 Combination Therapy of Stroke in Young Animals

All monotherapeutic attempts to prevent or ameliorate brain damage following stroke have failed so far. In view of previous findings indicating that stroke impacts a wide range of mechanisms, ranging from central nervous system (CNS) physiology over CNS regeneration and plasticity to the adaptive immune system in an age-dependent manner, the failure of monotherapies is perhaps not unlikely.

Tissue recovery after brain ischemia requires both neural and vascular components in the damaged brain area. The poor survival and differentiation of both the transplanted cells and their progenies in the inhospitable environment of the infarcted cortex have prompted the search for alternatives and new concepts like the neurovascular unit to limit the severe death of transplanted cells. Therefore, co-transplantation of NSCs with other cells in the niche may be a better strategy to improve microenvironment and make the grafting more efficient. To this end co-transplantation of neural stem cells and endothelial cells (ECs) within a mouse model of stroke enhanced the survival, proliferation, and differentiation of transplanted cells and improved functional recovery [126]. Taking a step further, co-transplantation of ESC-VPCs with NPCs after ischemic stroke supplied not only neural cells but also ECs and pericytes, thus providing nearly all important components for recovery of the neurovascular unit at the infarcted area [97].

The combination of mesenchymal stem cells and neural stem cells could improve also functional recovery after stroke if given prior to stroke. To this end, a mix of MSCs isolated from the femurs and tibias of rats and NSCs isolated from rat embryo ganglion eminence was labeled with PKH26-GL and administered one day before stroke into the lateral ventricle and neurological recovery evaluated for 28 days after stroke. The results indicate that the combination cell therapy is more efficient in promoting brain recovery after stroke than each stem cell alone [77].

More recently, triple cell co-transplantation with rat NSCs, astrocytes, and brain microvascular endothelial cells (BMECs) has been proposed. After grafting these cells into the brain of MACO/R rat model with different combinations, we found that the learning and memory ability of these rats improved to some extent. The rats which transplantation of NSCs with astrocyte and BMECs together have got the most achievement and they are better than those who grafted only two cells. The rats grafted only one cell have got the worst improvement. From our experiment, we get the conclusion that co-transplantation of NSCs with astrocytes and BMECs can improve learning and memory in the water maze test, probably due to the microenvironment improvement by the transplanted astrocytes and BMECs [33].

In an interesting approach, the combination therapy was given in sequence. First, mesenchymal stem cells were transplanted during the acute phase after stroke (1 day) in an attempt to diminish the inflammation and provide an appropriate microenvironment for regeneration after ischemia. Then, the neural stem cells were transplanted at 7 days after stroke to help regeneration by differentiation into neurons, oligodendrocytes, or astrocytes [77].

8.1.8 Co-administration of G-CSF and BMMNC in the Poststroke Aged Rats

Multimodal approach is an alternative strategy to promote functional recovery after stroke in aging brain, using systemic manipulation strategies. Several studies showed that grafting of autologous BM mononuclear cells (BM MNCs), a heterogeneous population containing mesenchymal and hematopoietic stem/progenitor cell fractions, in the peripheral circulation beneficially influences functional recovery as well as infarct volume in various species and is safe for stroke patients [21, 26, 161]. We therefore reasoned that timely application of BM MNC after stroke could bridge the gap until G-CSF-driven mobilization of endogenous BM cells comes to full effect, leading to improved outcome of this experimental combination treatment for stroke in aged rats.

G-CSF exerts a wide range of potential effects [120] and can reduce the number of fatal hemorrhages after experimental thrombolysis [46]. It has been suggested that G-CSF exerts a therapeutic effect after stroke by anti-apoptotic properties and by reducing excitotoxicity-driven penumbral apoptosis [163]. The latter effect was considered strong enough to reduce lesion size in young animals [24]. However, the aging brain is in need for increased glutamate signaling, which is reflected by abundant expression of Na^+-dependent membrane glutamate transporters, particularly in white matter areas. This in turn renders the aging brain highly susceptible to ischemic excitotoxicity, swiftly exhausting mitochondrial capacities [11]. Despite some positive impact of G-CSF on the aged brain apart from anti-excitotoxicity, this situation may have limited G-CSF monotherapy benefits [141] although a remaining benefit was clearly shown. We tested the hypothesis that treating poststroke aged rats with the combination of bone marrow-derived mononuclear cells (BMMNCs)

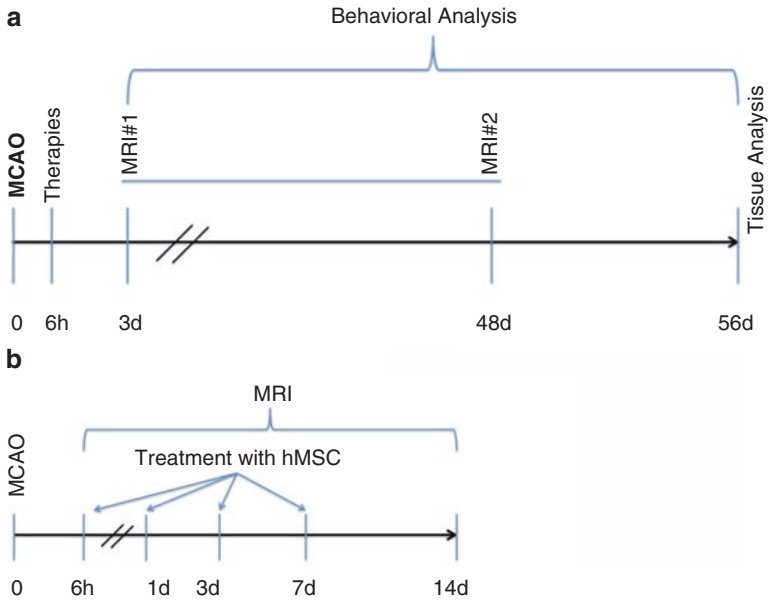

Fig. 8.1 Time window of BM MSC therapies. Most therapies start early after stroke with a single dose (**a**). The efficacy of several doses of cells given at different time points has also been tested (**b**). Current knowledge suggests that administered BMMNC provide indirect neuroprotection leading to infarct size reduction after ischemic damage in a time window of up to 1 month

and G-CSF improves the long-term (56 days) functional outcome by compensating the delay before G-CSF comes to full effect. To this end, 1×10^6 syngeneic BMMNC per kg bodyweight (BW) in combination with G-CSF (50 μg/kg, intraperitoneal application, continued for 28 days) was administered via the jugular vein to aged Sprague Dawley rats at 6 h poststroke (Fig. 8.1a). Infarct volume was measured by magnetic resonance imaging at 3 and 48 days poststroke and additionally by immunohistochemistry at day 56 (Fig. 8.1a). Functional recovery was tested during the entire poststroke survival period. Daily G-CSF treatment led to robust and consistent improvement of neurological function, but did not alter final infarct volumes. This result was unexpected since benefits of G-CSF and BMMNC treatment paradigms in stroke, independently from each other, have been repeatedly reported by independent experiments and groups and were hypothesized to work synergistically especially in the aged, stroke-lesioned brain. The combination of G-CSF and BMMNC did not further improve poststroke recovery. The lack of an additional benefit may be due to a hitherto not well-investigated interaction between both approaches and, to a minor extent, to the insensitivity of the aged brains to regenerative mechanisms. Also considering recent findings on other tandem approaches involving G-CSF in animal models featuring relevant comorbidities, we conclude that such combination therapies are not the optimal approach to treat the acutely injured aged brain.

Current knowledge suggests that administered BMMNCs provide indirect neuroprotection leading to infarct size reduction after ischemic damage in a time window of up to 1 month [93]. G-CSF in turn induces BMMNC mobilization, while the SDF-1/CXCR4 system causes BMMNC to invade the ischemic brain [164, 170], where they are believed to exert therapeutic effects. However, the initiation of this potentially beneficial effect may take simply too much time: although a granulocyte boost is seen after about 48 h, peaking of G-CSF-based mobilization can take up to 9 days [73], which is beyond the therapeutic time window for BMMNC. Since endogenous G-CSF is not available in sufficient concentrations directly after the ischemic event [24], a combination therapy providing (i) G-CSF in sufficient amounts to act neuroprotectively and (ii) exogenous BMMNC early enough to bridge the time gap until G-CSF-based endogenous BMMNC mobilization comes to full effect seemed promising – but failed to fulfill the expectations.

One may assume that either the lesioned and aged rat brain environment was insensitive to regenerative mechanisms by BMMNC or cell treatment has been mainly ineffective. Indeed, the aggravated impact of ischemic damage on the aged brain is well known, while potential detrimental effects of aging on BMMNC have been anticipated [136, 137, 185]. Moreover, technical complications may come in play as well: a limiting influence of long-term cryopreservation on the therapeutic efficacy of umbilical cord blood MNC, a population being very similar to BMMNC, has been discussed recently [191]. However, deriving syngeneic cells from young animals and limiting cryopreservation to no more than 4 weeks in our experiment might have limited such aging and cryopreservation effects on the donor side. Although a remaining impact cannot be excluded per se, a complete failure of the BMMNC treatment seems unlikely. An alternative explanation for the reduced efficacy of the combination treatment could be interference between both treatment regimes. A recent study in hypertensive animals demonstrated that intravenously administered BMMNCs occupy splenic granulocyte clearance capacities for apoptotic cells [143]. This clearance system usually removes apoptotic granulocytes from the circulation, which represents an important anti-inflammatory mechanism. Being already compromised by externally administered BMMNC, the swift and early granulocyte boost from the BM by G-CSF may have completely exhausted the clearance system in our treatment scenario. This detrimental interaction may have caused a sustained systemic and central pro-inflammatory bias, leading to subtle additional damage, not enhancing but partly reducing the neuroprotective G-CSF effect. It remains for further investigation whether this interaction is even more relevant in the aged brain.

False-negative results are a common phenomenon when selected sample sizes are too small to reveal small-scale treatment effects with G-CSF and G-CSF+BMMNC group outcomes just differing randomly from each other. To prevent such scenarios, we chose relatively large samples ($n=21$ animals per group). This is close to detect an effect size of 20 %, being recommended to assume when assessing experimental stroke therapies [111]. We therefore consider that insufficient study power is not very likely to have “masked” a positive effect of G-CSF+BMMNC.

8.1.9 Clinical Trials

Mobilization of hematopoietic stem cells by administration of granulocyte colony-stimulating factor has been assessed and not shown to have a therapeutic benefit in patients after stroke either [143, 154, 176]. Therefore the first goal of clinical trials was to assess the feasibility and safety of transplanting autologous bone marrow mononuclear cells into stroke patients [144, 145, 156, 161], and both positive [177] and negative [145] stroke outcomes have been reported. Recently, intravenous administration of autologous bone marrow mononuclear cells to patients with severe embolic stroke was shown to be feasible and safe with a tendency for improved neurologic recovery and improvement in cerebral blood flow and metabolism in poststroke [176].

8.1.10 Co-transplantation of G-CSF and BM MSC

Several studies showed that grafting of bone marrow-derived stem cells in the peripheral circulation improved functional neurological outcome and reduced infarct volume. Most of these studies used bone marrow mesenchymal cells (BM MSCs) [69, 124]. In addition, MSCs can serve as an excellent cellular delivery system. In a previous work, we have shown that application of G-CSF shortly after stroke in aged rats increases neurogenesis and improves some of the behavioral indices [141]. Even though hMSC S-TRAIL cells were seen within the injection site, a large number of cells migrated toward the tumor along the corpus callosum. In vitro, paracrine factors secreted by MSCs protect neurons from apoptotic cell death in the OGD model of cerebral ischemia [162].

Therefore we reasoned that the efficiency of the bone marrow-derived cell therapy may be increased by simultaneous application of G-CSF. In particular we tested the hypothesis that grafting of pre-differentiated bone marrow mesenchymal stem cells (BM MSC) in G-CSF-treated animals increases the odds of long-term functional outcome in aged rodents. To this end, 10^6 rat BM MSCs isolated from young Sprague Dawley rats were administered a single dose of BM MSCs (10^6/kg) given in combination with G-CSF (50 μg/kg for 28 days) via the jugular vein or intrathecally to Sprague Dawley rats at 6 h poststroke (Fig. 8.1a). The phenotypes of BMSCs used in this study were positive for CD105, CD166, CD29, and CD44. Cells tested negative for CD14, CD34, and CD45. Cells were tested for purity by flow cytometry and for their ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages. The control groups received daily injections of either G-CSF 50 μg/kg or vehicle (5 % glucose) for 28 days. To investigate the localization of injected cells, a separate group of aged animals were injected with mesenchymal cells of human origin (hBM MSCs). Although hBM MSCs are poorly immunogenic [38] and the rats survived just 4 days after administration, the animals were given cyclosporine A (s.c., Sandimmune, Novartis, 10 mg/kg) diluted in Cremophor EL (Sigma) to prevent graft rejection.

Infarct volume was measured by MRI at 3 and 48 days poststroke and additionally by immunohistochemistry at day 56 (Fig. 8.1a). Functional recovery was tested during the entire poststroke survival period of 56 days. Daily treatment of poststroke aged rats with G-CSF led to a robust and consistent improvement of neurological function. The combination therapy of GG-CSF+BM MSC in aged rats showed, surprisingly, no additional improvement in recuperation of the sensory function (adhesive tape), although recuperation of more complex motor (rotating pole) and spatial reference memory tasks was improved both by G-CSF and the combination. Paradoxically, MCAO rats swam slightly faster than unoperated animals, probably due to a poststroke excitatory state, an observation confirmed in previous studies [18].

However, of the treated groups the best recovery rate was seen for the G-CSF group which showed significant improvement in the water maze spatial reference memory task between days 21 and 42, suggesting that the beneficial effect of the G-CSF treatment is restricted to the G-CSF treatment period [141]. We hypothesized that the improved functional recuperation of the G-CSF group may have been helped by the stimulation of endogenous neurogenesis by G-CSF as previously reported [141]. In the combined treatment study, we found increased cellularity in the formerly infarct core of the G-CSF+BM MSC group at day 56 poststroke and intact neurogenesis in the lateral ventricle region. However, there was a clear regional separation of the DCX^+ cells which emanated from the ventricular wall and BrdU-labeled nuclei which were localized mainly in the vascular network of the lateral ventricle. Since BrdU was administered for the first 14 days after stroke, we hypothesize that at 2 months poststroke, DCX^+ cells with BrdU nuclei did not survive. Instead, $BrdU^+$ nuclei survived most likely in endothelial cells of the vascular wall [138]. Further, the combination therapy significantly improved recuperation and microvessel density in the formerly infarct core and beyond. Finally, we found that the aged brain environment is permissive for the migration of human BM MSCs toward the lesion site and concluded that, in a real clinical situation involving older poststroke patients, successful regenerative therapies may have to be delivered throughout a prolonged period, perhaps for 6–12 months.

8.1.11 Clinical Studies Using Combination Therapies

Novastem, a leader in regenerative medicine, treated stroke patients with ischemia-tolerant mesenchymal stem cells (itMSCs) in combination with ischemia-tolerant neural stem cells (itNSCs), both of which are proprietary products of Stemedica, given intrathecally to patients with motor aphasia due to ischemic stroke. The study aimed to evaluate functional changes on subjects at the baseline, at 90 days, and at 180 days, and it is measured using the United States National Institute of Health Stroke Scale (NIHSS), Stroke and Aphasia Quality of Life Scale-39 (SAQCOL-39), and the Boston Diagnostic Aphasia Examination (BDAE) neuropsychological evaluation for diagnosis. In addition, an MRI taken with a gadolinium-based contrast

agent (GBCA) is performed as a baseline analysis and at the study's conclusion. The end point is to evaluate the safety and tolerance of the two-cell treatment, as well as observing initial changes in functionality.

8.1.12 Therapeutic Window and Route of Administration for Combination Therapies

Several studies showed that grafting of bone marrow-derived stem cells in the peripheral circulation improved functional neurological outcome and reduced infarct volume [75]. Most of these studies used bone marrow mesenchymal cells (BM MSCs), but feasibility and safety in clinical trials were also shown for the bone marrow mononuclear cells (BMMNCs) [69, 124]. A conclusive result on optimal timing and dosing is, however, still missing.

The ideal therapeutic protocol in terms of number of cells and at what times to be delivered is uncertain. Different routes of MSC administration have been used to treat damaged ischemic brain tissue.

Numerous studies have shown that the intracerebral, intravenous, and intra-arterial routes of transplantation of stem cells are effective for ischemic stroke [12, 23, 26, 76, 93, 95, 121, 122, 188, 190]. The advantage of intracerebral transplantation is the targeted deposition of stem cells into the lesioned brain, compared to intra-arterial or intravenous transplantation resulting in cells being trapped in peripheral organs such as the spleen [1, 66, 184]. However, local stem cell delivery increases risks and side effects, such as bleeding and tissue injury. Therefore, in clinical practice, intravascular transplantation remains the preferred route of administration being much less invasive than intracerebral transplantation. Direct tissue injection via arterial administration may produce occlusion and embolization [49, 130, 168], while the intravenous approach would allow a broad biodistribution and easy access [66, 169]. Recent studies indicate that intra-arterial stem cell transplantation for ischemic stroke could induce functional recovery in ischemic stroke animals [26, 66, 79, 86, 89, 121, 157, 165, 166, 186], with a clinical phase I/II trial demonstrating its safety and feasibility in stroke patients [124].

The efficacy of mesenchymal stem cells (MSCs) to restore neurological function has been demonstrated repeatedly in animal models of stroke and might be a feasible and safe mode for treatment of stroke patients [39]. However, it is unclear how the timing of intra-arterial MSC transplantation to ischemic stroke affects the functional recovery. In a recent study intra-arterial allogeneic MSC transplantation in ischemic stroke model of rats at 24 h after MCAO led to robust functional recovery that was paralleled by reduced infarct volumes and a high number of integrated MSCs successfully migrating toward the ischemic cortex [182].

Omori and colleagues did a comparative study on the therapeutic benefits of multiple injections vs. single-dose application of therapeutic cells. Thus it was reported that a relatively large dose of 3×10^6 cells administered at an early time

point is more effective than multiple injections of 1×106 cells at 6, 24, and 48 h and showed nearly identical efficacy as a single-cell dose at 6 h and suggested that relatively high dosing of hMSCs within the first 2 days is the most effective protocol (Fig. 8.1b) [127].

In another study the beneficial effect of 3×107 bone marrow-derived mononuclear cells (BMMNCs) and BM MSC injected into the jugular vein after ischemia has been evaluated at 1, 7, 14, or 30 days poststroke in a rat model of focal ischemia induced by thermocoagulation [53]. The results indicate that the treatment of sensorimotor cortical ischemia with intravenously administered BMMNCs is only effective in the acute/subacute phases of the disease with only a few showing an improved functional recovery when MSCs were administered 1 month after ischemia [165]. Some other studies have shown that, on the contrary, intrathecal delivery by lumbar puncture may be a more efficient approach for the BM MSC treatment of stroke [101].

Some other studies have shown that intrathecal delivery by lumbar puncture may be useful and feasible for MSCs treatment of stroke [101]. However, we did not find major differences in the efficacy of the two routes of administration. In our model, CD166-positive cells in the ipsilateral hemisphere migrated all the way from the wall of the lateral ventricle (Fig. 8.2a) and its vicinity (Fig. 8.2b) through the corpus callosum (Fig. 8.2c) to the peri-infarcted region (Fig. 8.2d) and to the infarcted area (Fig. 8.2e). Noteworthy is the presence of human nuclei that were intermingled with rat nuclei in the infarcted area (Fig. 8.2f).

8.1.13 The Mechanisms by Which MSCs May Ameliorate Infarcted Brain Tissue

The mechanism of action of stem cell transplantation remains not fully understood and has been related more to the capacity of MSCs to release neuroprotective factors (paracrine mechanism) than to their capacity to replace damaged neural cells through their transdifferentiation properties. Administration of MSCs in acute stroke animal models markedly decreased brain infarct size, improved neurological function by enhancing neurogenesis, and showed anti-inflammatory and anti-apoptotic effects. Additionally, initial clinical studies using intravenously delivered MSCs have been initiated in human subjects with stroke [96].

Cellular therapy using mesenchymal stem cells (MSCs) can, in addition, enhance the endogenous restorative mechanisms of the injured brain by supporting processes of neovascularization and neurogenesis [12, 40, 42, 68, 78, 101]. Thus, beyond the formerly infarct core, several groups noted vigorous sprouting angiogenesis as evidenced by RECA/BrdU double-positive immunostaining of the blood vessels during the resolution phase of angiogenesis (Fig. 8.3). By number of laminin/BrdU co-localizations, the density of the newly formed blood vessels was significantly higher in the brains of aged animals treated with the combination G-CSF + BM MSC as compared to controls and G-CSF alone [10].

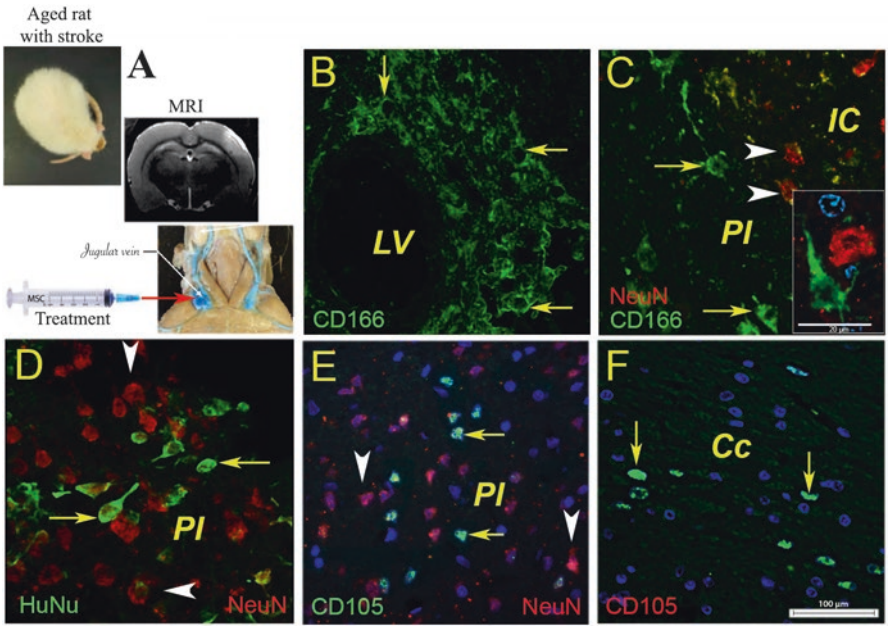

Fig. 8.2 Combination therapies of stroke. (**a**) Treatment with BM MSC starts early after stroke; (**b**) in our model the cells probably entered the injured brain via the lateral ventricle as shown by the CD166-positive cells; (**c**) about 1 % of the injected CD166-positive cells reached the infarcted area where they were intermingled with surviving or degenerating neuronal nuclei showing NeuN immunopositivity; (**d**, **e**) note the presence of immunopositivity for human nuclei (**d**; HuNu, *arrows*) and CD105 (**e**) that were dispersed between the rat nuclei in the infarcted area (NeuN; *arrowheads*); (**f**) vigorous sprouting angiogenesis has been reported as evidenced by RECA/BrdU double-positive immunostaining of the blood vessels (*violet*) as well as numerous $BrdU^+$ nuclei in the newly formed endothelium (*blue*) and reconstruction of the basal lamina (laminin, *green*) during the resolution phase of angiogenesis

These results strongly suggest that the BM MSC promoted angiogenesis rather than neurogenesis in the lesioned area of aged rats. Indeed, previous studies have shown that delayed intracerebral injection of hMSCs modified the cerebral microvasculature after transient ischemia [27, 122] and improved the cerebral blood flow (CBF) [82]. In our model, the MSC reached the peri-infarcted region after 4 days. However, 56 days after the administration of BM MSCs, the proliferation marker BrdU was incorporated preferentially in the "pinwheel" architecture of the ventricular epithelium [62, 99] and blood vessel in the formerly infarct core, while the DCX^+ cells occupied an adjacent, distinct position in SVZ and were not detectable at all in the lesioned area (Fig. 8.3). Since BrdU was administered for the first 14 days after stroke, we concluded that at 2 months poststroke, DCX^+ cells with BrdU nuclei did not survive, strongly suggesting that the early neuronal progenitors did not survive in the hostile environment of the poststroke aged brain. Instead, in animals treated with the combination of G-CSF and BM MSCs, we noted an increased number of newly formed blood vessels in the formerly infarct core and the

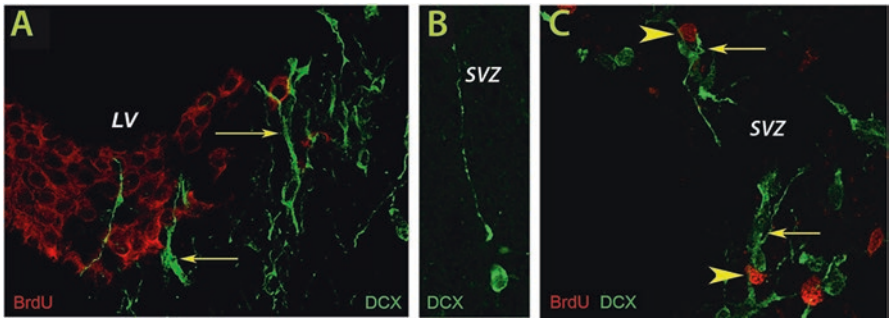

Fig. 8.3 Poststroke neurogenesis and angiogenesis following combined therapy, BM MSC with G-CSF. At 8 weeks poststroke, none of the DCX$^+$ cells in the SVZ of control animals co-localized with BrdU-labeled nuclei. Instead, the BrdU-positive nuclei were distributed mainly in the "pinwheel" architecture of the ventricular epithelium (**a**). The DCX$^+$ cells occupied an adjacent, distinct position (**a**, *arrows*). Some of the DCX$^+$ migrated away from the ventricular wall (**b**). We noted vigorous neurogenesis with many DCX$^+$ (*arrows*) co-localizing with BrdU nuclei in the G-CSF-treated animals (**c**; *arrowheads*)

region beyond it which we dubbed the "islet of regeneration" [30]. These results strongly suggest that BM MSCs promoted rather neurogenesis in the lesioned area [27]. Indeed, previous studies have shown that delayed intracerebral injection of hMSCs or intravenous administration of MSCs prepared from the bone marrow modified the cerebral microvasculature after transient ischemia [122] and improved the neovascularization along with enhanced functional recovery and cerebral blood flow (CBF) [82].

The current understanding of mechanisms underlying stroke treatment with bone marrow-derived cells is that homing of cells into the infarcted brain may cause trophic support and hereby enhanced poststroke recovery. Transplanted NSCs may promote recovery also without differentiating to neurons and even without long-term survival through several other mechanisms, e.g., neurogenesis [180], modulation of inflammation [76], neuroprotection [8], and stimulation of angiogenesis [27] and brain plasticity [105]. Delayed and persistent functional improvement induced by intracerebral NSC transplantation after stroke, without cell survival and tissue replacement at 6 months after the insult, was recently documented by electrophysiology, fMRI, and behavioral testing [149].

Therapeutic effects mediated by cell transplantation are likely related to the secretion of growth factors and cytokines. Mechanistically, studies done on young subjects using human umbilical cord blood-derived MSCs or bone marrow-derived cell treatment for stroke suggest that homing of cells into the infarcted brain may cause trophic support for host neurons and hereby enhanced poststroke recovery. Indeed, MSCs enriched from adult human umbilical cord and bone marrow have demonstrated therapeutic efficacy for treatment of stroke in a rat model, presumably by increasing the expression of cytokines CXCL2 and CXCL5 and growth factors BDNF, NT-3, FGF9, HBEGF, and VEGF in the ischemic brain that was

accompanied by reduction of the infarct volume, increased neurogenesis, and improved neurological function [12, 39, 42, 68, 78]. However, our combination therapy did not further enhance neurogenesis in SVZ of the aged animals.

Reports on the differentiation of transplanted cells yielded conflicting results. Earlier studies have shown that MSCs injected into the lateral ventricle of neonatal mice persistently engraft and migrate throughout the brain and adopt an astrocyte-, oligodendrocyte- [38, 108, 203], or endothelial cell-like phenotypes (ECs) some of them being incorporated into newly formed brain vessels [60, 71, 197]. Occasionally a neuronal phenotype has been reported in vivo [25, 94, 146] and in vitro [47] and by supporting axonal growth and provide tissue protection at sites of injury [4, 109]. Committed neural progenitor cells, NS-MSCs, produced in vitro from rat and human MSCs, differentiated into neuronal cells after transplantation and became immunoreactive to various neurotransmitter-related markers within the host tissue.

Neural stem cells (NSCs) originate from the ectoderm, and mesenchymal stem cells (MSCs) originate from the mesoderm; therefore, it is highly unlikely that stem cells isolated from one niche could form terminally differentiated cells from the other [159]. Nevertheless, recent data showed that undifferentiated BMSCs express not only mesodermal but also endodermal and ectodermal genes. Therefore, BMSCs are capable, in principle, of producing mesodermal derivatives and perhaps also ectodermal derivatives through transformation [103]. Further, during human neural development, the initiation of neuroectoderm begins with *Pax6* expression, followed by *Sox1*, which subsequently regulates the expression of downstream genes, such as *Nestin* [131, 198]. This knowledge led to generation of functional NSCs from hAD-MSCs by activating transcriptional factor for early neural development [59].

To this end BMSCs were transfected with the BDNF gene. The transfected BMSCs displayed a nerve-like phenotype and expressed the neural cell markers nestin and GFAP [3, 59].

Reports on the differentiation of transplanted cells yielded conflicting results. Earlier studies have shown that MSCs injected into the lateral ventricle of neonatal mice persistently engraft and migrate throughout the brain and adopt an astrocyte-, oligodendrocyte- [20, 108, 203], or endothelial cell-like phenotypes (ECs), some of them being incorporated into newly formed brain vessels [40, 60, 71, 197]. Occasionally, a neuronal phenotype has been reported in vivo [25, 94, 146] at the site of injury [4, 40, 109] and in vitro [47]. Committed neural progenitor cells, NS-MSCs, produced in vitro from rat and human MSCs, differentiated into neuronal cells after transplantation and became immunoreactive to various neurotransmitter-related markers within the host tissue.

It has been shown that MSCs are able to release several angiogenic and neurotrophic factors as well as anti-inflammatory molecules [48]. At this regard, it has been shown that when stimulated by inflammatory cytokines, MSCs increased their anti-inflammatory capacity, suggesting that MSCs may even improve their efficacy when localized in an inflammatory microenvironment in vivo [19].

8.1.14 Can Stem Cell Therapy After Stroke Limit Neuroinflammation?

The efficacy of all cell therapies so far is discouragingly low mainly because the time course of interactions between host neuroinflammation, which is considered to be a major obstacle to exogenous-mediated neuronal precursor cells, and stroke-induced neurogenesis or exogenously administered stem cells is virtually unknown.

Cell therapy itself can be used during the first week poststroke to limit neuroinflammation in animal models [8, 14, 52, 147]. Thus, syngeneic systemic delivery of mouse NSCs into mice subjected to stroke gave rise to an anti-inflammatory effect lasting up to 30 days [8].

A recent study emphasized the crucial importance of timing and cell dose for successful poststroke treatment using BM MSC. The study found that transplantation of BMSCs at 3 and 24 h, but not 7 days after focal ischemia, significantly reduced the lesion volume and improved motor deficits. Similarly, transplanted cells in the range $1 \times 10(6)$ to $10(7)$, but not at $1 \times 10(4)$ to $10(5)$, significantly improved functional outcome after stroke. In addition to inhibiting macrophage/microglia activation in the ischemic brain, BMSC transplantation profoundly reduced infiltration of gamma delta T (γδT) cells, which are detrimental to the ischemic brain, and significantly increased regulatory T cells (Tregs), along with altered Treg-associated cytokines in the ischemic brain [188]. Furthermore, the survival of MSCs was found to be very low (between 0 and 30%) and was highly dependent on the injection route/site (intravenous, intracerebral, or intrathecal) and cell source (auto-, allo-, or xenogeneic) [45]. Immune-related factors that impair neuronal survival and induce neuronal death also inhibit regeneration; therefore, immunomodulation should benefit stem cell therapy. Both in vitro and after transplantation in vivo, NSPCs not only form neural cells for replacement but also exert immunomodulatory and trophic effects, the so-called therapeutic plasticity [27, 92, 115].

Studies done in humans have largely confirmed animal studies. Thus implantation of human fetal NSCs into cortex or striatum after stroke may have immunomodulatory effects on the host tissue. Transplanted cells may act neuroprotectively by suppressing microglia/macrophage activation in the stroke-injured cortex both at 1 and at 6 and 14 weeks [51, 76, 118].

8.1.15 The Role of the Aged Microenvironment for the Combination Therapy

One conclusion from the heterochronic parabiosis studies is that the regenerative capacity of old tissue can be enhanced by the young systemic milieu [41]. However, it has been observed that rejuvenation of old tissue may require the neutralization or removal of inhibitory factors in the microenvironment [35, 36, 81, 194].

The aged brain is particularly refractory to growth phenomena after injuries. We and others have shown that potential mechanisms for self-repair also operate in the post-ischemic aged brain. The major factors involving the loss of regenerative capacity in the aged brain are an age-related decrease in neurogenesis and a loss of environmental hostility disturbing regeneration and migration of neuronal precursors toward the ischemic lesion due to the inflammatory response to stroke that is most evident in aged brains [136]. The current understanding of the involved mechanisms of bone marrow-derived cell treatment for stroke is that homing of cells into the infarcted brain may cause trophic support and hereby enhances poststroke recovery. Structural reorganization is mainly thought to be mediated by trophic factors released by the homed cells as well as neurotrophins [88, 163] and angiogenesis [115, 181, 197]. In addition, G-CSF obviously exhibits autocrine functions with anti-apoptotic and pro-differentiative effects in the brain [163].

The major factors involving the loss of regenerative capacity of the aged brain are an age-related decrease in neurogenesis and the environmental hostility created by the inflammatory response to stroke [139].

The microenvironment within the neurogenic niche allows the production of new neurons and provides permissive cues for their migration along the rostral pathway [160, 201, 202]. Since the neurogenic niche is localized around blood vessels [37, 166], the possibility arises that diminished neurogenesis during aging may be modulated by both CNS-derived cues [98, 123, 151] and cues extrinsic to the CNS delivered by blood.

In animal models, the number of new neurons in the injured striatum, a brain region located in close proximity to the neurogenic SVZ, in aged rodents after stroke was similar to that in young animals [2, 43] despite 50 % decline in neurogenesis in the subventricular zone of elderly rodents compared to young adult animals [57]. However, the proportion of surviving neurons is discouragingly low [6, 102, 132]. Similar findings have been reported in humans [83, 91, 110, 114]. Earlier studies on postmortem human brains provided evidence of cell proliferation and neuroblast formation after stroke even in aged patients, too [83, 110, 119]. The finding that new neurons are continuously added in the adult human striatum [58] along with the presence of an increased number of putative neuroblasts in the human striatum after stroke lends support to this hypothesis [110]. However, whether endogenous neurogenesis contributes to spontaneous recovery after stroke has not yet been established. In addition, age, comorbidities, physical condition of the patient, and severity of disease could substantially influence these steps and, therefore, the outcome of the healing process [135].

The process of cellular senescence can be an important additional contributor to chronic poststroke injuries by creating a “primed” inflammatory environment in the brain [29, 63, 84]. Persistent neuronal death causes a prolonged neuroinflammatory response in the infarcted area of comorbid subjects, too [70]. Previously we have shown that aged brains develop a fulminant inflammatory response to stroke [9]. The early phase of the inflammatory response (the first 10 days after injury) comprises infiltration of PMNs and T cells, and each cell population reaches its maximal abundance at specific time points. The second phase of cellular inflammation initiates

2 weeks after injury, peaks at 2 months, and remains detectable at 6 months post-injury [13]. Unfortunately, this time frame also coincides with the initiation of regenerative processes [28, 136]. Therefore poststroke inflammation may be one of the major factors that limits the efficacy of cell therapy especially with NPCs.

Although mild neuroinflammation can be beneficial for regenerative events aimed at functional restoration after stroke [133], persistent poststroke neuroinflammation results in decreased proliferation of the newly born NSPCs and ineffective integration into the circuitry of the reorganized brain area [106].

Earlier studies have suggested that neuroinflammation alone inhibits neurogenesis and that inflammatory blockade with indomethacin, a common nonsteroidal anti-inflammatory drug, restores neurogenesis after endotoxin-induced inflammation and augments neurogenesis after cranial irradiation [56, 125]. Recent studies have reported that targeting the inflammatory response to ischemic injury limits the expansion of the lesion and increases the survival of neurons after stroke [92, 112, 193]. For example, ibuprofen was found to downregulate the TBI-induced inflammatory response. In addition, migrating neuroblasts from transplanted cells were observed near the contusion and in the ipsilateral hippocampus in ibuprofen-treated animals only, suggesting that the anti-inflammatory treatment had beneficial effects on graft survival and/or differentiation especially in aged subjects [187].

In a recent study conducted on aged animals, the grafted hiPSC suppressed microglia/macrophage activation in the stroke-injured cortex as evidenced by differential morphological changes of these cells in the cell-grafted and vehicle-injected animals [179]. Although it is not clear how microglia/macrophages were affected at earlier time points after stroke in aged animals, it seems possible that the observed immunomodulatory action of the grafts could contribute to both neuroprotective and beneficial plastic responses in the host brain. Consistent with our findings, previous studies have shown that implantation of human fetal NSCs into the cortex or striatum after stroke can suppress the number of microglia/macrophages in the peri-infarcted area both at 1 [76] and at 6 and 14 weeks [118]. Importantly, also syngeneic systemic delivery of mouse NSCs into mice subjected to stroke gave rise to an anti-inflammatory effect lasting up to 30 days [8].

Recent experiments using both bone marrow-derived mesenchymal cells and neural precursor cells for stroke therapy suggest that the aged rat brain may not be refractory to cell survival and remodeling and that it also supports plasticity and remodeling. An open question remains, however, if transplanted cells have any beneficial effect on behavioral recovery. MSCs exhibit intrinsic homing properties to sites of injury, inflammation, and hypoxia [74, 113, 128, 173] that can be used for targeted delivery of therapeutic factors. In a mouse model of glioblastoma, a large number of cells migrated toward the tumor along the corpus callosum [117]. Our study shows that the aged rat brain environment still supports this migratory pathway in the ischemic brain as suggested by the presence of several markers of human MSCs (CD166 and CD105) in the corpus callosum and peri-infarcted area.

The combination therapy between G-CSF and stem cells in the present study did not fulfill all expectations. A potential explanation could be the fact that marrow stromal cells which may be mainly responsible for the supportive/trophic effect can-

not be mobilized by G-CSF treatment. Even more important could be the fact that G-CSF-induced stem cell mobilization from the bone marrow occurs typically with a delay of 6–9 days which was probably beyond the therapeutic time window for this approach [73, 107]. Nevertheless, the combination treatments did not show clear benefits for stroke in aged animals suggesting that either (1) G-CSF did not fulfill its chemoattractant role for the administered cells, or (2) one time cell administration is not sufficient to improve tissue recovery after stroke, or (3) the aged rat brain environment was hostile to regenerative events, or (4) the treatment was too short in duration.

8.2 Conclusions

To date, all monotherapeutic attempts to prevent or lessen brain damage following stroke have failed. In view of our findings that stroke impacts a wide range of systems in an age-dependent manner, from CNS physiology to CNS regeneration and plasticity, the failure of therapies aimed at only a single target system is perhaps inevitable.

To conclude, (1) the potential for neurogenesis is also preserved in aged, stroke-injured brains; (2) the environment of the aged brain is not hostile to transplantation of BM MSC; and (3) the extent of recovery is successful in some but not all behavioral tests [8, 28, 104, 179].

There remain significant developmental and translational issues to be resolved in future studies such as: (1) Understanding the differentiation into specific phenotypes. Upon transplantation, the differentiated cells often de-differentiate [85]. (2) Tumorigenesis remains a significant concern [153]. (3) Anti-neuroinflammatory therapies are a potential target to promote regeneration and repair in diverse injury and neurodegenerative conditions by stem cell therapy. (4) Efficacy of cell therapy can be enhanced by physical rehabilitation [55]. We recommend that in a real clinical practice involving older poststroke patients, successful regenerative therapies would have to be carried out for a much longer time. The BM MSC therapy in aged rodents warrants further investigation including repeated administrations of therapeutic cells at several time points after stroke and using various combinations with G-CSF or other relevant growth factors/cytokines.

Finally, a better understanding of potential risks of stem cell therapies in strokes hall makes the translation of cell therapies safer. Likewise, awareness of may help improve their efficacy to achieve therapeutic success [20].

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Chapter 9
Modification of Bone Marrow Stem Cells for Homing and Survival During Cerebral Ischemia

Yaohui Tang

Abstract Over the last decade, major advances have been made in stem cell-based therapy for ischemic stroke, which is one of the leading causes of death and disability worldwide. Various stem cells from bone marrow, such as mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), and endothelial progenitor cells (EPCs), have shown therapeutic potential for stroke. Concomitant with these exciting findings are some fundamental bottlenecks that must be overcome in order to accelerate their clinical translation, including the low survival and engraftment caused by the harsh microenvironment after transplantation. In this chapter, strategies such as gene modification, hypoxia/growth factor preconditioning, and biomaterial-based methods to improve cell survival and homing are summarized, and the potential strategies for their future application are also discussed.

Keywords Bone marrow stem cells • Modification • Homing • Survival • Ischemia

9.1 Introduction

Stroke is the third leading cause of mortality and the leading cause of long-term disability in the United States. Approximately 8,000,000 people suffer a stroke, and more than 140,000 people die each year. Ischemic stroke accounts for over 80 % of total stroke patients [126]. Though extensive neuroprotection and regenerative studies have been performed, only tissue plasminogen activator (tPA) has been proven to be effective. However, due to its narrow therapeutic time window (less than 4.5 h) and hemorrhagic complication, fewer than 5 % of stroke patients are able to benefit from tPA, and even among those, only 10 % return to independent living [82].

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Recently, growing evidence suggests that stem cells, including MSCs, neural stem cells (NSCs), EPCs, and induced pluripotent stem cells (iPS), are beneficial for cerebral ischemia [77, 112]. Among these, bone marrow-derived stem cells (BMSCs) have the most promising therapeutic potential, because a large quantity can be harvested autologously without ethical or immunological issues [120]. However, a number of problems remain unresolved and need specific attention prior to general clinical translation. For example, it is still challenging that stem cell survival, homing, and engraftment rates are low after transplantation in the pathological environment subjected to multiple insults, including ischemia/hypoxia, inflammatory response, and so on, which hamper the benefits and applications of cell-based therapy.

This chapter first summarized recent progress in basic and translational research in the field of BMSC transplantation for ischemic stroke. It then critically discussed how to enhance BMSC-based therapy by improving grafted cell survival and homing to further establish BMSC transplantation therapy as a scientifically proven method in clinical applications.

9.1.1 Basic Concept of Bone Marrow Stem Cells (BMSCs) in Stroke

Bone marrow (BM) consists of heterogeneous stem cell populations, including MSCs, HSCs, EPCs, and very small embryonic-like cells (VSELs). Their neuronal differentiation potential as well as neurotrophic factor secretion capacity has prompted interest in using BM as stem cell donor source for cell-based therapy in stroke.

9.1.1.1 Bone Marrow-Derived Mesenchymal Stem Cells (MSCs)in Stroke

BM-derived MSCs are a population of plastic-adherent fibroblastic cells, with CD29, CD105, and CD73 positive, but lack of hematopoietic surface markers such as CD34 and CD45. MSCs have the potential to differentiate into mesodermal cell lineages to involve in adipogenesis, chrondrogenesis, and osteogenesis [13]. MSCs derived from various donors, including rat, mouse, rabbit, or humans, have been transplanted by intravenous (IV), intra-artery (IA), intracerebral (IC), or intracisternal routes into animals, from different time points (hours to months) after induction of stroke, and have shown to improve functional recovery during cerebral ischemia [112]. Following IV and IC injection, MSCs migrate to the ischemic boundary; however, few cells have been shown to survive, and long-term cell engraftment has not been detected with IV administration [96]. Another study stated that only 3 % of administered cells expressed neuronal markers in vivo [15], which argued with the concept that tissue replacement is likely to be a potential mechanism for this

strategy. More studies support that trophic factors secreted by the MSCs in response to the local microenvironment stimulate endogenous neurogenesis, angiogenesis, and immunomodulation and further improve functional recovery. Higher levels of BDNF, NT3, and VEGF have been detected in the penumbra region 14 days after human MSC transplantation [5]. Increased VEGF and bFGF drive angiogenesis and facilitate regional blood flow [53]. In addition to secreting trophic factors, MSCs were also detected to influence astrocyte survival and astrocyte-related trophic factor expression after ischemic insult, by activating kinase pathways and protein functions [67]. Up to date, clinical reports also reveal that MSCs significantly improve patients' functional recovery without adverse side effects, probably through neuronal differentiation or secreting anti-inflammatory as well as neurotrophic factors [11].

9.1.1.2 HSC

Quiescent of CD34+ HSCs are able to migrate quickly from bone marrow to blood circulation in response to cerebral ischemia, which is induced by a wide array of chemokines and cytokines, including stromal cell-derived factor-1 (SDF-1) and granulocyte-colony-stimulating factor (G-CSF) [50]. During stroke, HSCs exit from bone marrow, migrate to the brain, adhere to the vascular wall, and cross over the blood–brain barrier (BBB), mediated by SDF-1/CXCR4 axis or G-CSF. This recruitment of HSCs from BM to stroke-induced lesion area has been employed in clinical protocols, for the creation of ample supply of HSCs for brain repair [10]. Both experimental and clinical studies have demonstrated the safety and feasibility of HSCs-based therapy for ischemic stroke. Intracerebral implantation of CD34+ HSCs promoted angiogenesis and neurogenesis and increased the local cortical blood flow in the ischemic hemisphere in ischemic rats [100] and mice [107]. A small clinical study demonstrated that by autologously IA injection of CD34+ cells into five stroke patients, all patients showed behavior recovery and infarct reduction, suggesting the potential of direct IA infusion of autologous CD34+ selected cells for the treatment of stroke [4].

9.1.1.3 EPC

In 1997, Asahara and coworkers first isolated Flk-1+/CD34+ cells from human peripheral blood, which were defined as EPCs. In that study they found that these cells could integrate into blood vessels when transplanted into a hind-limb ischemic mouse model [1]. EPCs were usually generated and maintained in bone marrow and could migrate into lesion region to help blood vessel remodeling and repair [71]. Recent studies showed that EPC transplantation could promote cerebral blood flow, reduce infarct volume and neuronal cell death, increase focal angiogenesis and neurogenesis, and improve neurobehavioral recovery after ischemia [26]. Grafted EPCs could either secrete neurotrophic factors, which is supported by the evidence that

EPC medium could also promote angiogenesis [144], or differentiate into endothelial cells to replace/repair injured ECs and integrate into endogenous blood vessels, which is detected by histological studies [26]. These results support that EPCs have great therapeutic potential for stroke, most possibly through both directly integrating into blood vessels and secreting trophic factors.

9.1.1.4 Very Small Embryonic-Like Cells

In 2006, Ratajczak's group first discovered a nonhematopoietic population that expresses neural lineage markers (GFAP, Nestin, Olig1, Olig2, Sox2, and Musashi-1) and resides in the nonhematopoietic CXCR4+/Sca-1+/lin-/CD45- BM mononuclear cell fraction, named as very small embryonic-like cells (VSELs) [90]. The number of circulating VSELs in PB increases in mice after experimental stroke [55] and in stroke patients [89], suggesting that VSELs residing in adult tissues or mobilized into PB are a potent source of adult tissue-derived stem cells that can be used for regenerative medicine, particularly for neural repair after stroke. Ratajczak et al. observed increased gene expression of both pluripotent and NSC markers in PB-borne nucleated cells in stroke patients, resembling what they previously noted in murine stroke model. Further analyses using computer tomography imaging revealed differences in VSEL mobilization between patients with posterior circulation infarcts and patients with partial anterior circulation infarcts [55]. In addition, the observation that murine VSELs are capable of differentiating into neurons, oligodendrocytes, and microglia further encourages us to use these cells as donor grafts for regeneration of a damaged CNS. However, a limitation for clinical application is the small number of VSELs that could be harvested, requiring ex vivo expansion strategy, especially to generate enough supply of VSELs for stroke therapy in clinical setting.

9.1.2 Mechanism of Death of Transplanted Stem Cells During Cerebral Ischemia

Although stem cell transplantation appears to be very promising for stroke, a number of problems remain unresolved and need specific attention in order to improve therapeutic efficacy for further successful clinical translation, including low survival and engraftment of transplanted cells in the brain subjected to multiple insults including ischemia, reactive oxygen species (ROS) generation, inflammatory response, apoptotic cascade activation, and so on.

Accumulating evidence demonstrates that less than 10% of transplanted stem cells could survive in the lesion site after transplantation as they are exposed in hostile environment, and cell death is initiated via multiple mechanisms [48]. It's reported that more cells survive when they are transplanted into sham animals (no brain injury) compared to injured animals [3], indicating that factors in the lesion

site induce death of the transplanted cells. These factors include but not limited to time after injury [47], distance from the transplantation site to the lesion site [86], state of the cells transplanted (differentiated or undifferentiated) [59], aging of the cells transplanted [108], host immune response [35], and phagocytic response of host [2]. Subsequent evidence shows that delivery time is the major determinant of the survival of transplanted stem cells. It is reported that NPC survival was significantly reduced following delayed cell delivery [20], which was mediated by the inflammatory milieu.

Cell death is initiated even prior to transplantation, explained by two main mechanisms: detachment of cells from adherent surface and the removal of growth factor, during the procedure of trypsinization and suspension. Inhibition of cell adhesion-induced cell death was first reported in 1994 by Frisch and Francis. They found that when epithelial cells were seeded in medium with the addition of soluble peptide-GRGDSP, which prevented cell attachment by blocking integrins, it resulted in increased apoptosis [28]. This kind of cell death is termed anoikis, which can be rescued by culturing cells on ECM-coated surfaces to promote cell adhesion. For example, oligodendrocyte progenitor cells cultured on glass coverslips coated with fibronectin or laminin showed greater viability compared to those cultured on non-coated surfaces [37]. In vitro study demonstrated that addition of laminin to neural progenitor cells increased the number of neurospheres and reduced cell death in comparison to control groups, while blocking the beta 1 integrin inhibited the effect of laminin, suggesting this is beta 1 integrin mediated [32]. One proposed explanation for detachment-induced cell death is that Bmf released from actin in terms of cytoskeleton stabilization is reduced after cell detachment. Bmf binds to Bcl-2 in mitochondria and neutralizes its antiapoptotic effect, which activates caspase-8, further releasing Bcl-2 from the mitochondria to induce cell death [29].

In addition to detachment-mediated cell death, removal of growth factors also induces apoptosis. Typically, c-Jun amino-terminal kinase (JNK) signaling pathway is activated when trophic support is removed, mediates c-Jun phosphorylation, thus induces the expression of proapoptotic factor-Bcl-2 family (DP5/Hrk). It further demonstrates that DP5 activates a proapoptotic member of the Bcl-2 family-Bax, causes mitochondrial damage, and releases cytochrome c, leading to the formation of apoptotic protease-activating factor 1 (Apaf-1)/caspase-9 complex, which activates caspase-3 resulting in cell apoptosis [138].

Therefore, cell–ECM interactions are reduced, and apoptosis is initiated even prior to transplantation when stem cells are trypsinized as single cells, cell survival is further reduced by needle insertion, and growth factors withdraw during the injection process, as well as hostile environment they confront in the lesion site after transplantation, given the generation of reactive oxygen species (ROS) and inflammatory response mediators in brain postischemia. It is highly accepted that cerebral ischemia caused excessive ROS would induce the apoptosis of the transplanted cells [12]. Our study showed that more than 80 % of grafted cells died within 72 h after administration [110], and our in vitro studies also suggested that exposure of stem cells to culture conditions which mimic the hostile environment in vivo (such as oxygen–glucose deprivation and H2O2 stimulation) led to the apoptosis mediated by ROS [110].

9.1.3 Strategies to Improve BMSC Survival

Both basic studies and clinical evidence strongly support that BMSCs could serve as a promising restorative therapy for stroke. However, as stated above, high stem cell death rate is the main hurdle that hinders the therapy. Scientists in the field propose several strategies to conquer this challenge, including gene modification, preconditioning, and biomaterial-based methods.

9.1.3.1 Gene Modification

After cerebral ischemia, both intrinsic and extrinsic apoptosis pathways are activated [3]. More than 80% of stem cells died after their transplantation, which is mainly caused by the activation of proapoptotic signals. Thus, downregulation of proapoptotic or upregulation of antiapoptotic cues by manipulating gene expression of stem cells posttransplantation may ameliorate the microenvironment and further enhance their survival. Indeed, overexpressing of Bcl-2 in embryonic stem cells (ESCs) increased their survival after injection into ischemic rat brain, as well as enhanced their neuronal differentiation, and improved functional outcome [123].

In addition to regulate apoptotic-related genes, amounting evidence shows that modification of trophic genes in stem cells also has significant impacts on their survival and therapeutic efficacy (Table 9.1). MSCs overexpressing BDNF or GDNF after injection into ischemic rats showed more cell survival, promoted functional recovery, and reduced ischemic damage at 7 and 14 days following MCAO, while rats that received CNTF- or NT3-transfected MSCs showed neither functional recovery nor ischemic damage reduction [57]. Liu et al. found that intravenously administered hMSCs overexpressing PIGF could accumulate in the ischemic lesions, further reduced lesion volume, enhanced angiogenesis, and elicited functional improvement [74]. FGF-2-modified MSCs with HSV-1 greatly reduced infarct volume and improved functional recovery at 14 days after stroke [40]. When surviving, a new apoptosis-inhibiting protein was overexpressed in MSCs and promoted MSCs' survival by 1.3-fold at 4 days and 3.4-fold higher at 14 days posttransplantation, which results in reduced infarct volume and improved neurological function [76].

Besides BMSCs, lots of studies from Dr. Kim's group showed that transplantation of human NSCs overexpressing BDNF [61], VEGF [60], or Akt-1 [61] could produce a two- to threefold increase in cell survival at 2 weeks and 8 weeks posttransplantation, as well as reduce infarct volume and improve functional recovery [171]. Transduction of NPCs with TAT-Hsp70 led to increased number of grafted NPCs, reduced BBB disruption, enhanced postischemic neurogenesis, and increased neurotrophic factor secretion [23].

During the last decade, microRNAs (miRs), a group of short RNA molecules that involve in posttranscriptional downregulation, have gained extensive attention in modulating cell survival. It is reported that miR-210 and miR-107 exert significant

Table 9.1 Enhancement of stem cell survival by gene modification

Ovexpressing genes	Stem cells	Transfecting agents	Stem cell fate	Therapeutic outcome
Bcl-2 [200]	Embryonic stem cells	Electroporation with Bcl-2 plasmid	Increased ES survival after injection	Into ischemic rat brain, as well as enhanced their neuronal differentiation and improved functional outcome
BDNF or GDNF [166]	MSCs	Adenovirus	More cells survival at 7 and 14 days following MCAO	Improved functional recovery and reduced ischemic damage at 7 and 14 days following MCAO
PIGF [175]	hMSCs	Adenovirus	More cells accumulate in the lesion area	Accumulate in the ischemic lesions, further reduce lesion volume, enhance angiogenesis, and elicit functional improvement
FGF-2 [160]	MSCs	HSV-1 vector	N/A	Reduced infarct volume and improved functional recovery
Ang-1 [184]	hMSC	Adenovirus	N/A	Rats receiving Ang-hMSCs exhibited comparable lesion reduction, improved functional recovery, and increased angiogenesis
Survivin [176]	MSCs	Lentiviral vector	Promote MSCs' survival by 1.3-fold at 4 days and 3.4-fold higher at 14 days posttransplantation	Reduced infarct volume and improved neurological function

(continued)

Table 9.1 (continued)

Ovexpressing genes	Stem cells	Transfecting agents	Stem cell fate	Therapeutic outcome
BDNF [61]	NSCs	Adenovirus	Threefold increase in cell survival at 2 weeks and 8 weeks post injection	Renewed angiogenesis, induce behavioral improvement in ICH animals
Akt-1 [61]	NSCs	Retroviral vector	50–100 % increased cell survival at 2 and 8 weeks posttransplantation	Induced behavioral improvement
Bcl-XL [170]	hNSCs	Retroviral vector	Number of hNSCs were 1.5-fold higher at 2 weeks and 10-fold higher at 7 weeks than controls posttransplantation	Improved locomotor scores and enhanced accuracy of hind-limb placement in a grid walk
HSP-70 [154]	NPCs; MSCs	TAT-Hsp70 protein transduction	Increased intracerebral numbers of grafted NPCs	Reduced BBB disruption, enhanced postischemic neurogenesis, and increased neurotrophic factor secretion; decreased apoptosis in the infarcted tissue and improved cardiac function
HSP 27 [180]		Lentiviral vector	Increased MSCs' survival in vitro and in vivo	
HGF [209]	MSC	HSV-1	N/A	Decreased apoptosis of neurons and reduced neurologic deficits and infarcts
CXCR4 [207]	MSC	Lentivirus	A significant increase in the number of eGFP-positive MSCs in the infarct areas	A reduction in the volume of the cerebral infarction and improved neurological function

(continued)

Table 9.1 (continued)

Ovexpressing genes	Stem cells	Transfecting agents	Stem cell fate	Therapeutic outcome
VEGF [169, 210]	NSC	Retroviral vector	2–3 fold increase in cell survival at 2 weeks and 8 weeks posttransplantation	Increased angiogenesis and behavioral recovery in mouse ICH model; improved focal angiogenesis and the Neurological Severity Scale score
	MSC	Plasmid transfection with lipofactamine	Improved cell viability	Enhanced the capillary formation in the infarction region and eventually attenuated left ventricular remodeling
		Facially amphipathic bile acid-modified polyethyleneimine (BA-PEI) conjugates [182]		

antiapoptotic effects in BMSCs by targeting caspase-8-associated protein-2 and programmed cell death-10 [81]. Pharmacological agents, including diazoxide [84], can induce protective miRs expression. Besides miRs, a recent investigation elucidated that preconditioning of MSCs with specific cell-free DNAs (cfDNAs) increased cell survival via Toll-like receptor 9 (TLR9) and translocation of nuclear factor-kappa B (NFkB) [54]. This evidence highlights the possibility that miRs and cfDNAs may be potential new targets to promote stem cell survival after transplantation.

Collectively, these exciting results suggest that gene modification is a promising strategy to increase cell survival after transplantation, and these enhanced cell survivals could contribute to reduced infarcts and improved behavioral recovery through neuronal differentiation and promoted trophic factor secretion.

9.1.3.2 Precondition-Based Method

Gene modification takes the risk that uncontrolled expression of introducing gene may have adverse effects and induce tumor formation on normal brain. Recent studies show that precondition strategy including hypoxia preconditioning, growth factor preconditioning, and antiapoptosis drug preconditioning could be a safe and efficient method [136]. Up to now, a number of sublethal insults including hypoxia [131], anoxia [119], hydrogen sulfide (H2S) [127], hydrogen peroxide (H2O2) [141], as well as growth factors, such as erythropoietin (EPO) [68], stromal-derived

factor-1 (SDF-1) [139], insulin-like growth factor-1 (IGF-1) [78], heat shock proteins (HSPs) [117], or pharmacological agents such as melatonin [110], minocycline [94], isoflurane [52], and lipopolysaccharide (LPS) [132], have been tested in stem cells (Table 9.2).

Sublethal hypoxia preconditioning applied to stem cells have shown to activate protective signals including hypoxia-inducible factor-1 (HIF-1), growth factors, Akt, and ERK signals to further enhance their resistance to apoptosis/necrosis cues by increasing survival signals [30]. Dr. Wei's group has performed extensive studies related to hypoxia preconditioning. In these studies they demonstrated that transplantation of hypoxia preconditioning MSCs improves infarcted heart function [38] and ischemic brain function [124] recovery via enhanced survival of implanted cells and angiogenesis. Also they found hypoxic precondition reduced ES-NPCs apoptosis by 40–50 % in serum-free medium via upregulation of erythropoietin (EPO), Bcl-2, and HIF-1alpha [114].

One study from Dr. Yang's group demonstrated that melatonin pretreatment increased MSCs' survival and proangiogenic activity through Erk1/2 signaling pathway [110], which is consistent with other studies that melatonin treatment enhanced adipose-derived mesenchymal stem cells (ADMSCs) survival and therapy for lung ischemia injury [134] and reduced grafted eEPC apoptosis/necrosis as well as increased their outgrowth in injured kidney [87]. For minocycline, Sakata et al. showed that transplantation of minocycline-preconditioned NSCs protected their survival from ischemic reperfusion injury via upregulation of Nrf2 and Nrf2-regulated antioxidant genes, increased their paracrine factors releasing, attenuated infarct size, and improved neurological performance [94], and doxycycline has the similar protective effects [80]. Additionally, low LPS pretreatment was found to protect MSCs against oxidative stress-induced apoptosis and increase cell engraftment after transplantation into ischemic heart [132]. A recent study showed that EPO pretreatment could also suppress MSCs' apoptosis in response to hydrogen peroxide stimuli [25].

9.1.3.3 Biomaterial-Based Method

The development of biomaterials has evolved from the first-generation, material-based approach that focused on mechanical strength, durability, and biocompatibility to the third-generation, bio-functional materials that try to integrate biological cues to modulate cellular functions by modifying with extracellular matrix (ECM) related to signaling molecules. In recent years, biomaterials have been proven to be an effective strategy for regulating cellular behavior, including promoting cell survival, directing cell differentiation. Advances in biomaterials engineering enable promoting grafted cell survival and engraftment and have generated much attention in stroke therapy.

With regard to injecting stem cells which are encapsulated within biomaterials into ischemic brain, the infarct cavity is always an ideal location. First, it is more clinical relevant since the transplantation procedure is not initiated until infarct cav-

Table 9.2 Preconditioning treatment to improve stem cell survival

Triggers	Stem cells involved	Animal model	Stem cell fate	Outcome
Hypoxia	hESCs [155]; MSC [158, 161, 201]; ES-NPCs [195]	Myocardial infarction; MCAO	Increased neural precursor cell survival; engraftment of MSC was increased; cell survival was increased; promoted their survival, migration, and homing to the ischemic brain region; promote transplanted cell survival	Promoted neuronal differentiation; improvement in global, regional, and diastolic left ventricular functions; an increase in angiogenesis, as well as enhanced morphologic and functional benefits of stem cell therapy; reduced infarct volume and improved behavioral recovery; ES-NPCs exhibited extensive neuronal differentiation in the ischemic brain, accelerated and enhanced recovery of sensorimotor function
Anoxia	MSC [172, 199]	Myocardial infarction	Increased cell survival	Increased fractional shortening, ejection fraction, arteriole density and decreased infarct size; increased the capillary density and the fractional shortening and attenuated myocardial fibrosis
Hydrogen sulfide	MSCs [202]	Myocardial infarction	Improved the survival rate of the transplanted MSCs	Reduced the infarct size and increased left ventricular (LV) function
IL-6	NSCs [94]	MCAO	Protected the grafted neural stem cells from ischemic reperfusion injury	Attenuated infarct size, improved neurological performance and angiogenesis
Melatonin	MSCs [181, 194]; ADMSC [205]	MCAO; acute lung ischemia-reperfusion injury; ischemic kidney	Improved survival of MSCs; decreased apoptosis of ADMSC	Reduced infarct volume, enhanced angiogenesis, neurogenesis and functional recovery; protected the lung from ischemic injury; increased survival, paracrine activity, and efficiency of MSCs
Minocycline	NSCs [188]; OPC [190]	MCAO; in vitro	Minocycline preconditioning protected the grafted NSCs from ischemic reperfusion injury; reduced apoptosis in response to OGD	Attenuated infarct size and improved neurological performance
Doxycycline	NSCs [178]	In vitro	Decreased cell death and increased cell viability after oxygen–glucose deprivation–reoxygenation	Showed cryoprotective via induced the expression of Nrf2
BDNF	NSC [186]	MCAO	Promoted cell survival 1 week after transplantation	BDNF pretreatment of NSCs results in higher initial NSC engraftment and survival, increased neuroprotection, and greater functional recovery

ity is formed, which is already 2–3 weeks after the onset of stroke; second, cavity is adjacent to the highly plastic peri-infarct region, and injection of stem cells into the cavity shows to achieve best outcome. Third, injection into the cavity will not damage normal brain tissues. Although directly injecting stem cells into infarct cavity shows its merit in reduced infarct volume, enhanced behavioral recovery, and increased angiogenesis and neurogenesis, low cell survival is still a major problem that hinders its clinical application. For instance, only 8 % of the grafted NSPC transplanted cells survived 4 weeks posttransplantation in Mongolian gerbils after focal ischemia [43]. In another study, approximately 4 % of grafted NPCs survived at 2 weeks posttransplantation [145].

Previous experimental studies showed that using Matrigel, fibrin glue gels, particles, and other scaffolds as matrices could improve the survival of stem cells in the infarct cavity posttransplantation (Table 9.3). Matrigel is an extracellular matrix comprised of ECM proteins and growth factor mixtures, including collagen, laminin, epidermal growth factor (EGF), and fibroblast growth factor 2 (FGF-2). Jin et al. injected NPCs encapsulated with Matrigel into the infarct cavity in both young and aged rats. Compared to control group, more cells were detected at the infarct site, and best functional recovery was achieved in the NPCs+Matrigel group [45, 46]. However, Matrigel is derived from a mouse sarcoma that raises a serious concern for its clinical application.

The functions of biodegradable polymers such as PGA and PLGA are also extensively investigated in stem cell-based therapy in stroke. For instance, Park et al. implanted NSCs seeded on polyglycolic acid (PGA), a high biocompatible scaffold into the infarct cavity, and found infarct volume was greatly reduced as well as establishment of neuronal connections between exogenous transplanted NSCs and endogenous neurons [85]. Modo's group demonstrated PLGA could act as a structural support for NSCs in infarct cavity to improve cell survival and function [7]. In their further study, they loaded VEGF into the PLGA microparticles and transplanted NSCs which were seeded on the VEGF-releasing PLGA particles into the cavity. Their results showed that VEGF-releasing PLGA not only provides structural support but also attracts ECs into the cavity to induce neurovascular formation [8].

Hyaluronan (HA), a glycosaminoglycan that naturally and abundantly exist in the brain, could involve in brain development and influence cell adhesion, migration, angiogenesis, and axon growth. Thus it is reasonable to choose HA as protective matrices to encapsulate cells for transplantation into the brain to maintain a hydrated and porous environment [83]. Recently, experimental studies from Dr. Thomas Carmichael's group proved that hydrogel composed of cross-linked hyaluronan and heparin sulfate significantly promoted NPCs' survival after transplantation into the infarct cavity, accompanied by reduced inflammation [145]. In their further study, they proposed to modify hyaluronic acid hydrogel with cell adhesion peptide RGD and cross-linked with either MMP degradable peptides or non-MMP degradable peptides through a Michael Addition reaction to produce two hydrogel formulations with two different stiffness moduli (300 Pa in MMP HA and 1000 Pa in non-MMP HA). NPCs derived from induced pluripotent stem cells (iPS-NPC) were encapsulated in the hydrogel matrix and delivered to the infarct cavity of stroke

Table 9.3 Enhancement of stem cell survival by biomaterials

Biomaterials	Stem cells involved	Animal model	Stem cell fate	Outcome
PLGA microparticles [147]	MHP36 cells	MCAO	Cell survival was increased, and cells were differentiated into neurons	PLGA microparticles acted as a structural support for NSCs;
PLGA microparticles loaded with VEGF [148]	hNSCs			NSCs showed neuronal differentiation, and neurovascular unit was performed in the infarct cavity
Matrigel [45, 162]	Human fetal NPCs	MCAO	Enhance the survival of transplanted NPCs	Behavioral recovery was improved, and infarct volume was reduced
Collagen [135, 167, 187, 203]	Cardiomyoblasts	Myocardial infarction	Enhanced early survival of H9c2 cardiomyoblasts after transplantation into ischemic hearts	Left ventricular function was improved
	NSCs	MCAO	Increased cell survival and distribution	Reduced infarct volume, induced angiogenesis
	Marrow stromal cell-derived NPCs;	Excisional wound healing model	Remained longer viability	Improved motor behavior;
	BM-MSCs			significantly enhanced angiogenesis and VEGF
Hyaluronan [168, 183]	NPCs; iPS-NSCs	MCAO	Promoted NPCs' survival and neuronal differentiation after transplantation into the infarct cavity	Enhanced neurovascular unit formation and reduced inflammation
Fibrin glue [151]	iPS	MCAO	N/A	Improved the motor function, reduced infarct size, attenuated inflammation cytokines, and mediated neuroprotection
Collagen with bFGF in gelatin microspheres [179]	NS-MSCs	MCAO	Increased cell survival and proliferation	Significantly improved histological and
				Functional recovery in the rat stroke model

mice. They found that hydrogel system with MMP and RGD modification promoted neuronal differentiation of iPS-NPC and induced minimum inflammation [58].

9.1.4 Bone Marrow Stem Cell Mobilization in Stroke

9.1.4.1 Factors Mediating BMSC Homing

Migration and homing of administered cells to the ischemic regions are clinically relevant and very critical to their therapeutic efficacy. A detailed analysis of the biological responses to brain injury would not only give us insight into the mechanism of stem cell homing but also give us important clues about how we can improve their homing capacity. Now it is clear that following brain injury, homing molecular cues, including chemokines, growth factors, and adhesion molecules, originating from the inflammatory zone in the injured brain, are activated and upregulated to cause BMSC homing. Chemokines such as G-CSF and SDF-1 have been demonstrated to be an important stem cell homing mediator that mobilizes stem cells from bone marrow into the PB. G-CSF treatment enhances tissue regeneration and improves recovery after stroke by mobilizing BMSCs from bone marrow into peripheral blood [91]. Previous studies showed that subcutaneous injection of G-CSF for 5 days after cerebral ischemia promotes BMSC migration to the lesion area, reduces infarcts, and enhances functional recovery in stroke rats [101]. G-CSF treatment is also demonstrated to facilitate neurogenesis in SVZ by increasing the infiltration of BMSC into the brain [99]. BMSCs exert their benefits on cerebral ischemic injuries through promoting neuronal repair and recovery of brain function, which provides a basis for the development of a noninvasive autologous therapy for cerebral ischemia. Some pilot clinical trials demonstrated that G-CSF could mobilize BMSCs in patients after acute stroke safely and provide better neurological outcome compared to conventional treatment [101].

SDF-1 is another important homing factor, which is secreted primarily by bone marrow fibroblasts and is required for BMSC homing/retention in the bone marrow microenvironment. SDF-1 and its receptors CXCR4 and CXCR7 were found upregulated after early focal cerebral ischemia [121] and showed beneficial for the adhesion and migration of BMSCs both to bone marrow and to ischemic tissue through activation of specific integrin molecules. Given that CXCR4 and CXCR7 are present on bone marrow stem cells [14], upregulation of SDF-1 in the local ischemic damage after injury may be related to stem cell homing and engraftment toward the injured tissue. During cerebral ischemia, SDF-1 was found primarily co-localized with endothelial cells and closely interacted with infiltrated BMSCs from bone marrow in the ischemic penumbra region, suggesting that SDF-1 may mediate trafficking of transplanted BMSCs to ischemically damaged tissue. Indeed, overexpression of SDF-1 in ischemic tissues has recently been found to augment EPC-induced vasculogenesis in hind-limb ischemic mice, as well as enhanced recovery of blood perfusion, increased capillary density, and induced partial incorporation of EPCs

into the microvessels [129]. Our previous studies have highlighted biphasic function of SDF-1 in stroke mice in a time-dependent manner. One study demonstrated that injection of CXCR4 inhibitor AMD3100 into ischemic mice during acute phase significantly suppressed inflammatory response and reduced blood–brain barrier disruption via inhibiting leukocyte migration and infiltration [39]; however, another study showed that overexpression of SDF-1 in mice brain during post-acute phase promoted neurovascular recovery, neurogenesis, and angiogenesis through enhancing migration of neural progenitor cells and endothelial cells, while AMD3100 reversed protective effects of SDF-1 [66].

In addition to chemokines, growth factors, inflammatory cytokines, and adhesion-related molecules also play important roles in stem cell homing. For instance, PDGF and VEGF are demonstrated to act as chemoattractants to induce migration of MSCs [105]; IL-6, (TGF)-β1, interleukin (IL)-1β, and tumor necrosis factor (TNF)-α stimulate chemotactic migration through matrix metalloproteinases (MMPs) secreted by the MSCs [18]. During the transmigration process of MSCs through the vascular endothelium, integrins and adhesion molecules are involved. Based on the fact that MSCs express α4β1 integrin and vascular cell adhesion molecule-1 (VCAM-1), it is proposed that MSCs roll along the vascular endothelium may share the same mechanism as white blood cells and HSCs to move through the blood vessels. Indeed, Ip et al. identified that β1 integrins are important for the intramyocardial traffic of MSCs by developing a functional genomics approach [42]. Moreover, the adhesion of rat MSCs to endothelial cells of microvessels is reduced by anti-VCAM-1 antibody [98].

9.1.4.2 Tracking of Grafted Stem Cells In Vivo

Different administration routes will result in different homing, distribution, and engraftment. Experimental studies demonstrated that intracerebral [], intra-arterial, intravenous, and intracisternal injection of MSCs result in reduced infarct volume and enhanced behavioral functional recovery, irrespective of pros and cons existing in each injection method [112]. Intracerebral injection delivers and had the highest cell retention in a desired location compared to other methods [111], but it also induces adverse effects involving seizures and transient motor function impairment given its invasive procedure. Intraventricular transplantation is less invasive but achieves less therapeutic efficacy as intraventricularly injected human NSCs into ischemic rat brain did not show improvement [102]. Intravenous delivery is safer and more feasible, but only few cells could localize to the infarct region [111]. Intra-arterial administration contributes to more cells retaining in the brain than intravenous delivery and is beneficial for behavioral recovery [63]. However, intra-arterial transplantation leads to high mortality (about 40 %) and morbidity due to cell accumulation and microemboli, especially when large-sized stem cells (e.g., MSCs) were transplanted intra-arterially [44], which is a major concern for its clinical translation.

In order to determine stem cell migration and in vivo distribution, noninvasive and real-time imaging modalities are developed in recent years. Several multifunctional nanoprobes with high MR sensitivity are developed by our group to label stem cells and allow us to longitudinally track them after injection by MRI in terms of its high spatial resolution. In one study we labeled MSCs [122] and NSCs [142] with high MR sensitivity fluorescent-magnetite-nanocluster (FMNC) and tracked them by MRI and fluorescent imaging after injection into the contralateral hemisphere of the ischemic mice brain. MSCs were detected to migrate toward the perifocal region of the ipsilateral hemisphere through the corpus callosum. We further developed a trifunctional nanoprobe by adding iodine-125 to superparamagnetic iron oxide nanoparticles, which allows us to quantitatively track MSCs injected into the brain by micro-SPECT/CT and MRI. Using this method we found 30 % of intracerebrally grafted MSCs migrated from the injection hemisphere to the lesion area, and intravenously injection induced more than 90 % of MSCs migrated and accumulated in the lung, while no cells were found in the brain (Fig. 9.1) [111]. However, one major limitation of SPIO-based imaging strategy is that survival and dead cells cannot be distinguished. Signals from survival and dead cells are all captured by MRI and micro-SPECT. To resolve this problem, bioluminescence imaging (BLI) is developed and widely used to track the migration and survival of transplanted cells which are modified with a firefly or Renilla luciferase (Luc) enzyme [143]. However, the spatial resolution and the penetration depth of BLI are limited, which hinder its clinical application at current stage.

Recently, radionuclide probes for PET imaging were designed, as 18F-fluorodeoxyglucose ([18F]-FDG) is the most popular one. Several studies have reported direct imaging of transplanted cells with 18F-FDG [9, 113]. To track survival of grafted cells, the herpes simplex virus type 1-derived thymidine kinase (HSV-1-tk), which could exclusively phosphorylate substrates composed of acycloguanosines, is employed and routinely used to monitor human ESCs and C17.2 NSCs in the rodent brain [106, 118].

9.1.5 Strategies to Improve Bone Marrow Stem Cell Homing

Stem cell homing is a multistep process involving cell attachment, adhesion to the vascular endothelium, and migration through the tissue stromal, which are mediated by different factors, including chemokines, growth factors, integrins, and adhesion molecules. Understanding the mechanism of homing could help us to develop novel strategies to improve their homing ability and further increase the therapeutic efficacy. In principle, those methods that used to increase stem cell survival could also apply to improving stem cell homing.

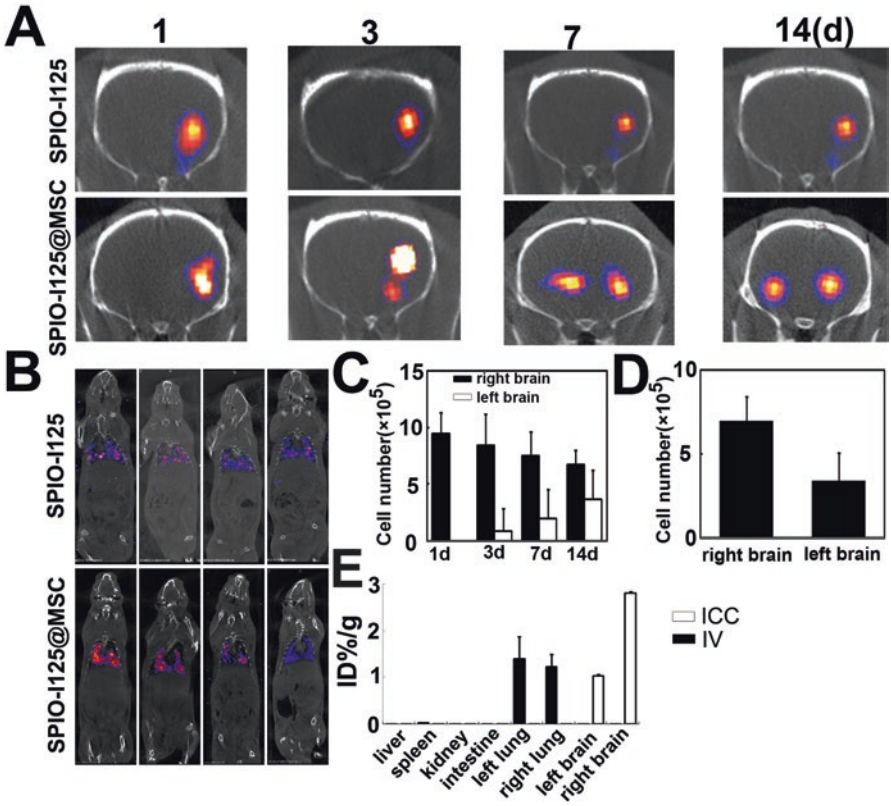

Fig. 9.1 SPECT/CT tracking of (125) I-fSiO4@SPIO-labeled MSCs in ischemic rats after IC and IV injection. (**a**, **b**) SPECT/CT imaging of labeled MSCs and particles alone in ischemic rats after IC (**a**) and IV injection (**b**). (**c**) The radioactivity detected in the right and left hemispheres accounting for the total transplanted dose at different time points after IC injection. (**d**) Ex vivo analysis of radioactivity in right and left hemispheres accounting for the total transplanted dose 14 days after IC injection. (**e**) Biodistribution of (125) I-fSiO4@SPIO-labeled MSCs at 14 days after IV or IC transplantation (Reprinted from Tang et al. [111], Copyright 2015, with permission from Wiley)

9.1.5.1 Homing Gene-Based Method

We and others demonstrated that genetic modification of the target tissue or the stem cells with homing genes is feasible to stimulate their homing ability and further improve behavioral recovery after stroke (Table 9.4). By stereotactic injection of adeno-associated virus (AAV) carrying SDF-1α gene into ischemic mice brain, Li et al. found that migration of endogenous neural stem cells and OPCs from subventricular zone to the peri-infarct region was enhanced and induced increased neurogenesis and oligodendrogenesis, reduced brain atrophy, as well as improved white matter and behavioral recovery [66, 69].

Table 9.4 Enhancement of stem cell homing by gene modification

Overexpressing genes	Stem cells	Animal model	Outcome
CXCR4 [207]	MSCs	Myocardial Infarction	Increased accumulation of BMSCs in the lesion area and an improvement in cardiac function
CCR1 [159]	MSC	Myocardium infarct	CCR1-MSCs accumulated in the infarcted myocardium at significantly higher levels. CCR1-MSC-injected hearts exhibited a significant reduction in infarct size, reduced cardiomyocyte apoptosis, and increased capillary density
ACE2 [150]	EPC	Cerebral ischemia	ACE2 overexpression improved the abilities of EPC migration and tube formation, reduced cerebral infarct volume and neurologic deficits, increased cerebral microvascular density and angiogenesis
HGF siRNA [149]	ASC	Hind-limb ischemia	Transduced ASC-shHGF secreted >80 % less HGF, which led to a reduced ability to promote survival, proliferation, and migration of mature and progenitor endothelial cells in vitro
IGF-1 [156]	MSC	Permanent coronary artery occlusion	IGF-1 transgene expression induced massive stem cell mobilization via SDF-1α signaling and culminated in extensive angiomyogenesis in the infarcted heart
GDNF [163]	NPC	Stroke	More NSPC-GDNF cells migrated toward the ischemic core, reduced infarct volume, and improved behavioral recovery
SCF [193]	NSPCs	Normal mice	Recombinant SCF induces potent NSPC migration in vitro and in vivo through the activation of c-kit on NSPCs
MicroRNA 9 [153]	hESC-derived neural progenitors	Stroke	hNPCs without miR-9 activity also showed enhanced migration

In addition to SDF-1 α, Yu and coworkers demonstrated its receptor CXCR4 also plays a pivotal role in stem cell homing. By transducing MSCs with CXCR4 by lentivirus and injecting them via the femoral vein following MCAO, they found that CXCR4 overexpression promoted MSCs' migration to the infarct region and enhanced neuroprotection via increased angiogenesis [137]. Besides stroke, MSCs overexpressing CXCR4 was also proved to migrate into the cardiac infarct area in a cardiac infarct animal model, leading to a significant improvement in cardiac func-

tion [17]. Additionally, growth factors also show their capacity to enhance stem cell migration posttransplantation. Haider et al. demonstrated that IGF-1 overexpression promoted MSC recruitment through paracrine activation of SDF-1α and enhanced myocardial repair [31]. When NPCs overexpressing GDNF were injected into ischemic rat brain, more cells were found accumulated in the lesion area [49]. For EPC it was reported that overexpression of angiotensin-converting enzyme 2 (ACE2) improved the EPC migration and tube formation, and injection of lentivirus-ACE2-transfected EPCs reduced cerebral infarct volume and neurological deficits, which was driven by eNOS [16].

Recently, miRNAs were demonstrated to play an important role in stem cell migration. One study from Delaloy et al. for the first time identified miR-9 as a novel regulator that coordinates the proliferation and migration of hNPCs. They found that hNPCs without miR-9 activity showed enhanced migration when transplanted into mouse embryonic or adult brains in a stroke mouse model [21]. Other miRNAs such as miR-10b and miR-204 have been also proven to play an important role in cell migration [41, 72].

9.1.5.2 Preconditioning-Based Method

As we discussed above, although overexpression of homing genes in both grafted stem cells and local brain tissues improves stem cell homing, several disadvantages exist in this strategy. For instance, uncontrolled expression of introducing genes raises the safety issue, and the risk of tumorigenicity such as leukemia also limits its application. Recently, upregulation of homing genes in MSCs under stress conditions including hypoxia has been confirmed, which may be mediated by HIF-1 alpha [24]. It is reported that hypoxia induces CXCR4 and CXCR7 expression in BMSCs via upregulated HIF-1α [75], and hypoxia preconditioning enhances migration of MSCs via increased expression of cMet [93], which hints at the possibility that hypoxia preconditioning could enhance mobilization of stem cells to lesion sites in ischemic brain. In addition to hypoxia preconditioning, H2O2 preconditioning could increase the migration of MSCs through upregulation of CXCR4 and activation of extracellular signal-regulated kinase (ERK) [65], and pretreatment of HSCs with SDF-1 or dextran sulfate enhances their homing to bone marrow, which is involved in several genes including CXCR4 and MMP-9 [33].

Accumulating evidence shows that pretreatment with growth factors also increases MSCs' mobilization (Table 9.5). In previous investigations, IGF-1 as well as VEGF increased MSC migratory responses via CXCR4 chemokine receptor signaling which is PI3/Akt dependent [70, 109]. Early studies have demonstrated that statins increased EPC number and function through activating the Akt/eNOS pathway [22]. Likewise, enhancement of eNOS enhancers improves the stem cell homing. In particular, pretreatment with eNOS enhancers significantly increased the homing of the intravenously infused EPCs or BMCs and led to increased exercise capacity in a hind-limb ischemia model [95].

Table 9.5 Preconditioning mediators to enhance stem cell homing

Triggers	Stem cells involved	Animal model	Stem cell fate	Outcome
Hypoxia	hESCs [155] MSC [158, 161, 201]; ES-NPCs [195]	Myocardial infarction; MCAO	Increased neural precursor cell survival; engraftment of MSC was increased; cell survival was increased; promoted their survival, migration, and homing to the ischemic brain region; promote transplanted cell survival	Promoted neuronal differentiation; improvement in global, regional, and diastolic left ventricular functions; an increase in angiogenesis, as well as enhanced morphologic and functional benefits of stem cell therapy; reduced infarct volume and improved behavioral recovery; ES-NPCs exhibited extensive neuronal differentiation in the ischemic brain, accelerated and enhanced recovery of sensorimotor function
H2O2	MSC [172, 199]	Myocardial infarction	Increased cell survival	Increased fractional shortening, ejection fraction, arteriole density and decreased infarct size; increased the capillary density and the fractional shortening and attenuated myocardial fibrosis
Hydrogen sulfide	MSCs [202]	Myocardial infarction	Improved the survival rate of the transplanted MSCs	Reduced the infarct size and increased left ventricular (LV) function
IGF-1	NSCs [94]	MCAO	Protected the grafted neural stem cells from ischemic reperfusion injury	Attenuated infarct size, improved neurological performance and angiogenesis
VEGF	MSCs [181, 194]; ADMSC [205]	MCAO; acute lung ischemia-reperfusion injury; ischemic kidney	Improved survival of MSCs; decreased apoptosis of ADMSC	Reduced infarct volume, enhanced angiogenesis, neurogenesis, and functional recovery; protected the lung from ischemic injury; increased survival, paracrine activity, and efficiency of MSCs
Minocycline	NSCs [188]; OPC [190]	MCAO; in vitro	Minocycline preconditioning protected the grafted NSCs from ischemic reperfusion injury; reduced apoptosis in response to OGD	Attenuated infarct size and improved neurological performance

(continued)

Table 9.5 (continued)

Triggers	Stem cells involved	Animal model	Stem cell fate	Outcome
Doxycycline	NSCs [178]	In vitro	Decreased cell death and increased cell viability after oxygen–glucose deprivation–reoxygenation	Showed cryoprotective via induced the expression of Nrf2
BDNF	NSC [186]	MCAO	Promoted cell survival 1 week after transplantation	BDNF pretreatment of NSCs results in higher initial NSC engraftment and survival, increased neuroprotection, and greater functional recovery
Valporate and lithium [198]	MSC	MCAO	Priming with VPA or lithium increased the number of MSC homing to the cerebral infarcted regions, and copriming with VPA and lithium further enhanced this effect through VPA-induced CXCR4 overexpression and lithium-induced MMP-9 upregulation	Priming with VPA and/or lithium improved functional recovery, reduced brain infarct volume, and enhanced angiogenesis

9.1.5.3 Biomaterial-Based Method

With the rapid development of tissue engineering, many state-of-the-art biomaterials have been developed to combine stem cells to treat cerebrovascular diseases, with the ultimate goal of repairing organs and tissue. In past two decades, many protein-based, polysaccharide-based, polymer-based, peptide-based, and ceramic-based scaffolds that have been proven to promote the viability, differentiation, and migration of stem cells are well designed [125]. Both natural and synthetic biomaterials have been developed and combined with stem cell-based therapy to promote cell survival and migration posttransplantation (Table 9.6).

Fibrin gel is ranked as the first biomaterial to prevent bleeding and promote wound healing in terms of the abundance of fibrinogen, ease fabrication, controllable gelation time, and tunable mechanical property. Fibrin gel is able to exclusively enhance the migration of the transplanted cells toward the lesion boundary zone, even it disappears completely 4 weeks after transplantation [133]. In one study performed by Lee and coworkers, they designed a VEGF-releasing gel that could attract NSC migration [171]. It is also reported that PEGylated fibrin patch controlled the release of SDF-1α at the infarct site and increased the rate of c-kit+ stem

Table 9.6 Biomaterial-based method to enhance stem cell homing

Biomaterials	Stem cells/ homing factors involved	Animal model	Stem cell fate
Fibrin gel	BMSC [204]	Cortical injury	Fibrin matrix enhanced the retention of the transplanted cells within the lesion, migration toward the lesion boundary zone, and differentiation into the neurons and perivascular cells
	C17.2 cell line [171]	Myocardial ischemia	The cells migrated toward the fibrin gel, with the total migration distance of 102.4 ± 76.1 μm over 3 days
	(PEGylated) fibrin patch [208]		The myocardial recruitment of c-kit+ cells was significantly higher in the group treated with the SDF-1a PEGylated fibrin patch
Alginate microspheres	Bone marrow-derived progenitor cells [165]	Hind-limb ischemia	Increased mobilization of bone marrow-derived progenitor cells and also improved recruitment of angiogenic cells expressing CXCR4 from bone marrow and local tissue
	hMSCs [206]	Myocardial ischemia	RGD-modified alginate improved cell attachment and growth and increased angiogenic growth factor expression
starPEG-heparin hydrogels	EPCs [146]	In vitro	Higher migration rates were achieved
Gtn-HPA hydrogels and PCNs	NPCs [174]	In vitro	Gtn-HPA/SDF-1-PCN hydrogels promoted hemotactic recruitment to enhance infiltration of aNPCs by 3- to 45-fold relative to hydrogels that lacked SDF-1
Collagen microgel	hMSCs [197]	Hind-limb ischemia	Optimized hMSC embedded microgels were shown to induce vascular repair and functional improvement by increasing SDF-1 expression
HA	EPCs [177]	Myocardial ischemia	Induced continuous homing of EPCs and improved left ventricular function in a rat model of myocardial infarction
	SDF-1 [191]		Injection of biomimetic hydrogels containing SDF-1 and Ac-SDKP increased stem cell homing and significantly improved left ventricle function, increased angiogenesis, decreased infarct size and great

(continued)

Table 9.6 (continued)

Biomaterials	Stem cells/ homing factors involved	Animal model	Stem cell fate
PLGA	SDF-1 [152, 196]	In vitro	Released SDF-1α caused significant migration of MSCs throughout the duration of release from the microspheres
			Threefold increase of the host-derived stem cell migration at the interface for up to 2 weeks
PCL	MSCs [189]	Bone tissue engineering model	MSCs were shown to migrate within a polycaprolactone scaffold in response to SDF-1
PLEOF [157]	BMSCs	In vitro	The migration of BMS cells in response to time-released SDF-1alpha closely followed the protein release kinetics from the hydrogels
PUASM [164]	SDF-1	MCAO	Systemic administration of SDF-1α-loaded copolymer into ischemic rat resulted in enhanced angiogenesis and neurogenesis
SPIONs combined with exterior magnet	EPCs [173]	MCAO	SPION-labeled EPC homing was greatly increased in ischemic hemisphere with magnetic field treatment
	MSCs [185]	Balloon angioplasty in a rabbit model	Magnetic targeting of mesenchymal stem cells gives rise to a sixfold increase in cell retention following balloon angioplasty in a rabbit model
	hNSCs [192]		Magnet treated rats had a larger number and greater distribution of ferumoxide-labeled NSCs as compared with controls

cell recruitment and offered potential therapeutic benefits in the myocardium ischemic mice [140]. This body of work suggests that migration of stem cells can be monitored by fibrin scaffolds.

Recently, scaffolds fabricated from gelatin [130], collagen [88], alginate [36], and hyaluronic acid (HA) [128] have been developed for the controlled release of growth factors, which could provide homing signals to enhance stem cell migration. Kuraitis et al. found encapsulating SDF-1 into alginate microspheres led to increased mobilization of bone marrow-derived CXCR4+ progenitor cells and restoring perfusion to ischemic tissues via neovascularization [56]. Further studies demonstrated hMSCs encapsulated in RGD-modified alginate microspheres are capable of facilitating myocardial repair [135]. Baumann et al. reported that encapsulating SDF-1α with starPEG-heparin hydrogels enhanced migration of EPCs in vitro [6]. Lim et al.

developed a multifunctional biomaterial comprising injectable gelatin-hydroxyphenylpropionic acid (Gtn-HPA) hydrogels and dextran sulfate/chitosan polyelectrolyte complex nanoparticles (PCNs) to carry SDF-1 to promote infiltration of NPCs through MMP-9 [73]. In particular, an interesting study fabricated and optimized a shape-controlled 3D type-I collagen-based microgel platform to modulate SDF-1 expression of hMSCs, and hMSCs embedded in the microgels were shown to induce vascular repair and functional improvement in hind-limb ischemic mouse [116]. Currently, HA is gaining its popularity as a biomaterial for tissue regeneration [62]. By chemically modifying HA with hydroxyethyl methacrylate, controlled release of SDF-1 was achieved after its encapsulation into HA, and enhanced endothelial progenitor cell chemotaxis was identified [79]. It is also reported that loading SDF-1 and angiogenic peptides (Ac-SDKP) to HA-based hydrogel promoted regeneration of cardiac function through increasing stem cell homing and angiogenesis [103].

Poly lactic-co-glycolic acid (PLGA) is an FDA-approved polymer and the most attractive polymeric drug/protein carrier among those synthetic materials as its high biocompatibility, biodegradability, and tunable mechanical property. PLGA has been extensively designed for controlled release of small molecule drugs, proteins, and other macromolecules in commercial use and in research. Double-emulsion solvent extraction/evaporation is a routine technique to load proteins to biodegradable PLGA microspheres. Using this strategy, Cross et al. loaded SDF-lα into PLGA microspheres for releasing SDF-1α over 50 days without affecting its bioactivity, and significant migration of MSCs throughout the duration of release from the microspheres was observed [19]. Thevenot and colleagues fabricated PLGA salt-leached scaffolds to carry SDF-1 and implanted in the subcutaneous cavity of Balb/c mice. They found this strategy enhanced host-derived stem cell engraftment by threefold compared to conventional mini-osmotic pump delivery for up to 2 weeks with limited inflammatory response [115].

In addition to PLGA, polycaprolactone (PCL) and poly (lactide ethylene oxide fumarate) hydrogel (PLEOF) have also been used to achieve MSC recruitment. Schantz et al. have developed acellular PCL scaffolds that allowed sequential delivery of VEGF, SDF-1, and bone morphogenetic protein-6 (BMP-6) in the rat and increased MSCs infiltrating into the scaffold, with concomitant angiogenesis [97]. In another study, He et al. synthesized SDF-1-loaded PLEOF hydrogel with poly(l-lactide) (PLA) fractions. A pronounced burst release followed by a period of sustained release was achieved, and MSCs showed migration to SDF-1 in a dose-dependent manner [34]. Recently, Kim et al. synthesized a dual pH-sensitive copolymer-poly (urethane amino sulfamethazine) (PUASM)-based random copolymer for controlled release of SDF-1 in stroke. This copolymer showed high protein encapsulation efficiency at pH 7.4, and at pH 5.5, it could release protein rapidly. Systemic administration of SDF-1α-loaded copolymer into ischemic rat resulted in enhanced angiogenesis and neurogenesis [51].

Recent studies have highlighted the role of superparamagnetic iron oxide nanoparticles in targeted cell delivery. Experimental studies from Dr. Yang's lab showed that intravenous injection of SPION-labeled EPCs into ischemic mice and

followed by magnetic field treatment promoted their migration to the infarcts, further reduced brain atrophic volume, and improved neurobehavioral outcomes [64]. Other studies with this method also showed that magnetic targeting of MSCs or hNSCs led to increased cell retention following their injection [92, 104]. An interesting study reported that small direct current (DC) electric fields induced significant directional migration of hNSCs toward the cathode independent of CXCR4 signal [27].

9.2 Conclusion

Bone marrow-derived stem cells have been demonstrated as promising sources of adult stem cells for regeneration and repair of neurological disorders, including ischemic stroke. On the other hand, many experimental studies make us recognize many fundamental questions related to the cell survival, homing, and engraftment that contribute to the limited efficacy of BM-derived stem cell transplantation in the clinic. We and other groups have proposed many strategies such as gene modification, preconditioning treatment, and biomaterial-based method to overcome these limitations. Strategies to improve cell survival and homing would enhance their therapeutic efficacy and strengthen the application potential of stem cell therapy. In summary, stem cell-based therapy for ischemic stroke in humans is still in its infancy. Further basic and translational studies are required before it becomes a scientifically proven strategy in clinical setting.

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Chapter 10
Clinical Studies of Bone Marrow-Derived Stem Cell Therapy in Stroke Patients

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Abstract Stroke is the leading cause of long-term disability in adults and the third cause of mortality worldwide. In the very acute phase of stroke, thrombolytics and endovascular thrombectomy can reduce stroke disability; however, only a small minority of patients receive these treatments. Once the neurological deficits are established, there are few options for recovery. In recent years, extensive cell therapy preclinical research has demonstrated a neurorestorative effect after cerebral ischemia. In cerebral ischemia animal models, bone marrow-derived stem cells improve neurological outcomes even in the long term, increasing brain plasticity and enhancing recovery mainly due to secretion of growth factors and cytokines.

In the bone marrow, different types of cells have been used for cell therapy in stroke. The first type of cells used for stroke and the most extensive studied in preclinical research are mesenchymal stem cells (MSCs). In recent years some other cells have been studied for stroke therapy with promising results, such as bone marrow mononuclear cells (BM-MNCs), hematopoietic stem cells (HSCs), and multipotent adult progenitor cells (MAPCs). Several phase I and II clinical trials have been published to date with these stem cells, which have already demonstrated the feasibility and safety of this therapy in the stroke setting. An increasing number of

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clinical trials, mainly with bone marrow MSCs and BM-MNCs, are ongoing to further assess the best dose, route, and timing of this therapy and to elucidate the efficacy cell therapy in stroke.

Keywords Patients • Bone marrow-derived stem cells • Therapy • Stroke

10.1 Introduction

The World Health Organization (WHO) defined stroke as a "rapidly developing clinical signs of focal (or global) disturbance of cerebral function, with symptoms lasting 24 h or longer or leading to death, with no apparent cause other than of vascular origin" [1].

Annually, 15 million people worldwide suffer a stroke and, in the United States alone, a person dies every 3 min due to stroke. In high-income countries, stroke is the third most common cause of death, only after coronary heart disease and cancer. Stroke is also the main cause of acquired adult disability [2]. Of those who survive a stroke, five million people annually are left permanently disabled, placing a burden on family and society. Only 41 % are independent 6 months after a stroke.

The ischemic stroke represents 80–85 % of strokes and the incidence is 150–200 patients/100,000 per year [3]. Stroke prevalence is about 2 % of the population of >20 years; however, it increases to up to 6–7 % in older adults (>65 years). Projections show that by 2030, an additional 3.4 million people in the United States aged ≥18 years will have had a stroke, a 20.5 % increase in prevalence from 2012 [4]. In the EU, the cost attributed to stroke was €64.1 billion in 2010, mainly due to the high costs of long-term special care and rehabilitation [5]. Moreover, the socioeconomic burden of stroke is expected to increase due to aging of the population and the rise in diabetes and obesity, which are reaching an epidemic level.

The currently available therapies of acute stroke target rapid vessel recanalization, since, without restoration of cerebral blood flow, hypoperfused cerebral tissue in the penumbral region progresses to cellular death that ultimately expands the necrotic core lesion. Nowadays, thrombolytics (i.e., tissue plasminogen activator or tPA) and endovascular thrombectomy are the main therapies for restoring normal perfusion in acute ischemic stroke. However, tPA has important limitations, with a narrow therapeutic window of 4.5 h, which means that less than 5–10 % of ischemic stroke patients receive this treatment. Moreover, recanalization rates after the administration of tPA are low and prevent disability in only 55 patients per 1000 people treated, without reducing mortality [6]. Recently, endovascular thrombectomy has demonstrated efficacy in several clinical trials in those patients with a large vessel occlusion [7]. Although the treatment approach of acute ischemic stroke is rapidly evolving [8], recanalization therapies are only administered to a minority of acute stroke patients.

Another approach to improve outcomes in stroke is the administration of neuroprotective drugs. Neuroprotective treatment aims to reduce the damage of stroke, but as most of the injury occurs in the first 24–48 h, these therapies must be administrated soon after stroke onset, and to date, no drug has been demonstrated to ameliorate the disability or mortality after stroke [9, 10].

In recent years, many studies have shown that once the stroke is established, profound neurorestorative processes are induced in brain tissue in response to focal cerebral ischemia [11]. Although these processes are insufficient to restore neurological function, neurorestorative treatments with pharmacological or cell-based therapies could stimulate and amplify these endogenous mechanisms in stroke patients. This approach has the major advantage of a wider therapeutic window, as neurorestorative therapies can be instituted during the recovery phase of the stroke, and promotes the remodeling of brain tissue. This makes the treatment available to a much larger number of stroke patients.

Also, neurorestorative treatments target not only the ischemic and "penumbra" tissue (hypoperfused tissue) but also viable brain tissue with normal perfusion stimulating neuronal plasticity and neurological recovery [11]. However, until now, neurorestorative drugs targeting single steps in the cascade of cerebral ischemia have failed to improve neurological deficits, probably related to stroke complexity, with necrosis, apoptosis, inflammation, and remodeling occurring as a continuum.

Stem cell therapy, such as transplantation of bone marrow stem cells, represents one of the most exciting fields in regenerative medicine and has emerged as an attractive approach for the treatment of stroke. These stem cells are believed to exert multiple therapeutic actions. They might target simultaneously several processes by releasing different factors inducing neuroprotection and brain remodeling and modulating the post-ischemic inflammatory response [12–15]. Extensive basic research has been done during the last two decades in cell therapy and stroke animal models, but we are still in the first steps in the clinical research with stroke patients.

The potential of cell-based therapy relies on several *key properties*: (1) their capacity to differentiate into several cell lineages, (2) their immunomodulatory properties, (3) their ex vivo expansion potential, (4) their ability to secrete factors to regulate biological functions such as proliferation and differentiation over a broad target of cells, and (5) their ability to home to damaged tissues.

10.2 Bone Marrow Cell Therapy and Clinical Trials

To date, there are many different cells being investigated for stroke, both in preclinical studies and in clinical trials such as embryonic stem cells, neural stem cells, adipose-derived stem cells, induced pluripotent stem cells (iPS), and stem cells obtained from bone marrow, umbilical cord, and amniotic or placental tissue. However, in this chapter, we will focus on bone marrow cell therapy as most of preclinical and clinical studies have used bone marrow stem cells [16, 17].

There are several advantages of using bone marrow stem cells as a cell therapy for stroke. First, the efficacy and reproducible benefits of these cells have been demonstrated in several laboratories and in different animal stroke models. Second, bone marrow stem cells are adult cells and therefore do not have ethical problems, unlike fetal and embryonic cells. Third, it has been proven reliable to use bone marrow stem cells in different time periods of stroke, even in the acute stroke phase. Finally, although some studies have been done with allogenic cells, bone marrow stem cells allow autologous administration avoiding the possibility of rejection.

In the bone marrow, different types of cells have been used for cell therapy in stroke. The first type of cells used for stroke and the most extensive studied in preclinical research are mesenchymal stem cells (MSCs). In recent years, some other cells have been studied for stroke therapy with promising results, such as bone marrow mononuclear cells (BM-MNCs), hematopoietic stem cells (HSCs), and multipotent adult progenitor cells (MAPCs).

10.2.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs or marrow stromal cells) are one of the first types of cells that have been studied for ischemic stroke. MSCs have a considerable therapeutic potential that has generated markedly increasing interest in a wide variety of biomedical disciplines. Extensive preclinical studies with MSCs have made this therapy a very promising cell-based approach for stroke.

MSCs are multipotent adult stem cells defined as those cells which have three characteristics: (a) must be plastic adherent when maintained in standard culture conditions; (b) must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14, or CD11b, CD79alpha, or CD19 and HLA-DR surface molecules; and (c) must at least differentiate to osteoblasts, adipocytes, and chondroblasts in vitro [18]. The lack of expression of HLA-DR (class II major histocompatibility complex) gives them an immunoprivileged status, and also their relative ease of isolation from bone marrow makes these cells a good candidate for cell therapy in different illness such as stroke.

The safety of MSCs has been analyzed in a recent meta-analysis of clinical trials with more than 1000 patients in different clinical conditions that included ischemic stroke, Crohn's disease, myocardial infarction, cardiomyopathy, and graft versus host disease. An excellent safety profile of MSCs was demonstrated, as there was no association between MSC treatment and acute infusional toxicity, organ system complications, infection, death, or malignancy [19]. However, larger controlled clinical trials are required before defining a definitive safety profile of MSCs.

Regarding efficacy outcomes, MSCs have demonstrated efficacy in clinical trials in other conditions such as graft versus host disease and are under study in autoimmune diseases (i.e., Crohn's disease, multiple sclerosis, and type 1 diabetes) and different models of ischemia (i.e., stroke, ischemic cardiac diseases, and limb ischemia) [20–22].

In animal stroke models, injection of MSCs resulted in very large and favorable effects on neurological outcomes. In a recent meta-analysis of preclinical studies, MSCs improved consistently multiple outcome measures with very large effect sizes. These data are robust across species studied, administration route, dose, and presence of comorbidities [23]. Furthermore, MSCs attenuated tissue damage and migrated into the ischemic boundary zone accompanied by reduced neuronal apoptosis and enhanced neoangiogenesis and synaptogenesis [24, 25].

The first clinical trial published with bone marrow stem cells in stroke patients was done by Bang et al. which used autologous MSCs [26] (Table 10.1). In this placebo-controlled phase I/II trial of 30 patients with chronic stroke, 5 patients were treated with MSCs and 25 were controls. Those patients in the treated group received two doses of IV autologous mesenchymal stromal cells at 4–5 weeks and 7–9 weeks from the onset of symptoms. This method was reportedly safe and feasible in the short term. In 2010 Lee et al. published the long-term follow-up, with 52 patients finally included (16 in MSC group and 36 in control group). No MSC-related adverse events were reported, with significant improved neurologic recovery in those patients receiving cellular therapy compared to controls (the proportion of patients with modified Rankin scale score 0–3 increased in the MSC group, $p = 0.046$) [27].

Other pilot trials have been published using MSCs in stroke. Honmou et al. reported IV MSCs transplantation in 12 patients with chronic ischemic stroke [28]. No adverse events were described from transplantation, and interestingly, a reduction of more than 20 % of infarction volume was observed in magnetic resonance imaging at 1 week after cell injection.

In another trial, Bhasin et al. published a trial including 40 chronic stroke patients [29]. Of these, 14 patients received intravenous BM-MNCs, 6 patients received intravenous MSCs, and 20 were control patients. During follow-up, stem cell transplantation was reported to be safe, and there was a significant improvement at 6 months in the Barthel index when the whole stem cell group was compared to the control group, although there was no difference in Rankin scale or Fugl-Meyer scale.

In spite of these previous experiences with MSCs, there are several disadvantages of using MSCs in stroke patients (Table 10.2):

1. MSCs require several weeks of cell culture to obtain sufficient quantity of cells for transplantation, not allowing the autologous injection of MSCs in the acute-subacute phase of stroke.
2. The large size of cells (13–19 μm) could also lead to pulmonary entrapment when administered by intravenous injection or even to microvascular occlusions and new cerebral infarctions in the intra-arterial (IA) route [30].

To date, there is no published data of allogenic transplantation of bone marrow MSCs or with a different route than intravenous but a very recent interim report of an open-label single-arm study of surgical transplantation of modified bone marrow-derived mesenchymal stem cells [31]. In this interim analysis of 16 patients that have completed 12 months of follow-up, authors describe significant improvement in NIHSS (National Institute of Health Stroke Scale) (mean decrease 2.00 [95 %

Table 10.1 Published clinical trials with bone marrow-derived stem cell therapy in stroke patients

Cell population	Author	No. of patient treated (controls)	Design	Route	Cell dose	Time window	Follow-up (month)	Adverse events
MSCs								
Autologous	Bang (2005) [26]	5 (25)	Randomized, observer-blinded phase I/II	Intravenous	5×10^7	4–5 weeks	12	None reported
Autologous	Lee (2010) [27]	16 (36)	Observer-blinded phase II	Intravenous	5×10^7	5 weeks	60	None study-related reported
Autologous	Honmou (2011) [28]	12 (0)	Open-label phase I	Intravenous	$6–16\times10^7$	36–133 days	12	Mild fever, nausea, appetite loss
Autologous	Bhasin (2013) [29]	6 (6)	Open-label phase I/II	Intravenous	$5–6\times10^7$	3–24 months	6	None study-related reported
Allogenic	Steinberg (2016) [31]	18 (0)	Open-label phase I/II	Intraparenchymal	$2.5–10\times10^6$	6–60 months	12	Headache, nausea, subdural hematoma (2 of 18)
BM-MNCs								
Autologous	Suarez-Monteagudo (2009) [41]	5 (0)	Open-label phase I	Intraparenchymal	$1.4–5.5\times10^7$	1–10 years	12	Headache, drowsiness, nausea, fever
Autologous	Barbosa (2010) [60]	6 (0)	Open-label phase I	Intra-arterial	$12.5–50\times10^7$	<90 days	6	None study-related reported
Autologous	Battistella (2011) [43]	6 (0)	Open-label phase I	Intra-arterial	$10–50\times10^7$	2–3 months	6	Seizures (2 of 6)
Autologous	Savitz (2011) [46]	10 (0)	Open-label phase I	Intravenous	$7–10\times10^6$/kg	24–72 h	6	None study-related reported

Autologous	Friedrich (2012) [44]	20 (0)	Open-label phase I	Intra-arterial	2.2×10^7	3–7 days	6	None study-related reported
Autologous	Moniche (2012) [47]	10 (10)	Observer-blinded phase I/II	Intra-arterial	16×10^7	5–9 days	6	Seizures (2 of 10)
Autologous	Prasad (2012) [49]	11 (0)	Open-label phase I	Intravenous	$0.2–18\times10^7$	7–30 days	6	One reinfarction
Autologous	Li (2013) [42]	60 (40)	Observer-blinded phase I/II (hemorrhagic stroke)	Intraparenchymal	$0.2–2\times10^7$	5–7 days	6	Fever, one unspecified pulmonary tumor
Autologous	Rosado-de-Castro (2013) [45]	12 (0)	Open-label phase I	Intravenous ($n=5$), intra-arterial ($n=7$)	$10–50\times10^7$	19–89 days	6	Seizures (7 of 12)
Autologous	Prasad (2014) [50]	85 (35)	Blinded randomized phase II	Intravenous	28×10^7	18.5 days	12	None reported
Autologous	Sharma (2014) [59]	24 (0)	Open-label phase I/II	Intrathecal	1×10^6/kg	40 months	30	None reported
CD34+								
Autologous	Banerjee (2014) [16]	5 (0)	Open-label phase I	Intra-arterial	$1.2–2.5\times10^6$	<7 days	6	None study-related reported
MAPC								
Allogenic	Hess (public presentation)	65 (61)	Blinded phase II	Intravenous	40×10^7	24–48 h	6	None study-related reported

Table 10.2 Comparative of bone marrow mononuclear cells and bone marrow mesenchymal cells

Type of cell	Advantages	Disadvantages
BM-MNCs	Consistent beneficial effect and an excellent safety profile in animal models	Not allow allogenic transplantation without immunosuppressive drugs
	Excellent safety profile in pilot clinical trials in stroke	Variability in the number of cells obtained after bone marrow harvest
	Prepared for administration within hours	
	No tumor formation	
BM-MSCs	Very large and favorable effects in stroke models	Require cell culture (several weeks)
	Immunoprivileged status, allow allogenic transplantation	No allow autologous administration in acute stroke patients
	Excellent safety profile in other clinical conditions and pilot stroke trials	Large size of cells that could lead to microvascular occlusions or pulmonary entrapment
	No tumor formation	

confidence interval, −2.7 to −1.3; $P<0.001$]) and Fugl-Meyer scale (mean increase 19.20 [95 % confidence interval, 11.4–27.0; $P<0.001$]). Patients included had a chronic ischemic stroke (mean 22 months from stroke onset) and received a stereotactic injection of allogenic MSCs (*SB623 cells*) in the peri-infarct area. Serious adverse events were unrelated or unlikely to be related to cell treatment. Postsurgery headache was the most common adverse event that was probably or definitely related to the procedure, experienced by 77.8 % of patients, and subdural hematoma and epileptic seizure were detected in two patients (11 %).

Although the immunoprivileged status of MSCs makes a rejection of allogenic transplantation very unlikely, its safety has to be proven in stroke patients. However, previous reports of allogenic use of MSCs in other conditions described no acute infusional toxicity [19]. Regarding the route, the probably main reason for the absence of MSC clinical trials using intra-arterial route is due to the potential of arterial embolism that has been described with animal stroke models, even in mammalians. Lu et al. [30] described an intra-arterial MSC transplantation in a canine stroke model with the development of new infarctions 24 h after transplantation in 16 % of dogs, probably due to impeded cerebral blood flow [32].

Due to the large and favorable effects in preclinical studies and in spite of the disadvantages described, MSCs are still one of the best candidates for cell therapy in stroke patients, and several clinical trials are currently ongoing.

10.2.2 Hematopoietic Stem Cells

Other bone marrow stem cells that have been investigated in animal stroke models are hematopoietic stem cells (HSCs). These cells express CD34 (CD34+ cell) and can be also found in peripheral blood and umbilical cord blood. Preclinical studies of CD34+ cells have shown significant benefits in animal stroke models, with

evidence of functional improvement as well as reduced infarct volume [33]. In a preclinical study, intravenous CD34+ cell transplantation resulted in increased perilesional angiogenesis and subsequent neurogenesis in mice at 48 h post-stroke [34]. There are also other evidences of neurogenesis and angiogenesis induced by CD34+ cells in subacute stroke, with cells transplanted expressing neuronal, glial, and vascular endothelial cell markers [35]. There are some preliminary clinical trials demonstrating the safety of autologous CD34+ peripheral blood stem cells [36, 37]. Bone marrow hematopoietic stem cells have only been used in a pilot open-label clinical trial of five stroke patients [38]. CD34+ cells were collected from the bone marrow of the subjects before being delivered by catheter angiography into the ipsilateral middle cerebral artery within 7 days from stroke onset. No safety issues were described and all patients show improvement of neurological deficit during follow-up, although no comparison was done with a control group. Authors found a nonsignificant reduction in the mean lesion volume from inclusion to day 180, with no new lesions in MRI (edema, hemorrhage, or tumor). To date, very few clinical trials are currently ongoing testing bone marrow CD34+ cells in stroke patients.

10.2.3 Bone Marrow Mononuclear Cells (BM-MNCs)

BM-MNCs are one of the most studied types of cells for use as stroke therapy. BM-MNCs are composed of a mixture of myeloid, lymphoid, erythroid, and stem cell populations, which includes HSCs, MSCs, and endothelial progenitor cells. The main advantage over other types of cell therapy is that autologous transplantation is feasible, even in the acute phase of stroke, as they are isolated from bone marrow and prepared for administration within hours. As MSCs, BM-MNCs have been extensively studied in animal models demonstrating a consistent beneficial effect and an excellent safety profile. Several biological effects such as attenuation of neuronal death, modulating microglia, reducing pro-inflammatory responses, increasing neoangiogenesis, and promoting proliferation of endogenous neural stem cells have been invoked [12, 39, 40]. However, few clinical studies have assessed the safety and efficacy of BM-MNC transplantation in stroke patients.

The first trial published with BM-MNCs was an open-label trial with five stroke patients treated with intraparenchymal route by Suarez-Monteagudo et al. [41]. Patients included had a chronic stroke from 1 to 10 years from onset and authors describe an excellent tolerance of procedure and with no important adverse events derived from surgery or transplant. After this study, only Li et al. have published a clinical trial using intraparenchymal route, although not including ischemic stroke but intracerebral hemorrhage (ICH) [42]. In this study, autologous BM-MNCs were injected to the perihemorrhage area in the base ganglia through an intracranial drainage tube 6 days after ICH. Surgical drainage of ICH was performed in every patient within the first day from ICH onset, and after 5 days those patients who consent to be treated with BM-MNCs were included in the study group ($n=60$), and those who rejected cell therapy were the control group ($n=40$). Both groups had

similar baseline characteristics and similar NIHSS (National Institute of Health Stroke Scale) after surgery, but authors describe a significant improvement in Barthel and NIHSS scores in the study group 6 months after inclusion (57.39 ± 23.51 in study group vs 46.90 ± 20.29 in control group, $P < 0.01$ in Barthel scale and 10.09 ± 8.86 vs 14.35 ± 10.14, $P < 0.01$ in NIHSS).

Since the trial published by Suarez-Monteagudo et al. in 2009, several clinical trials have been published with BM-MNCs. Most of them used less invasive routes as intravenous or intra-arterial injection.

A Brazilian trial published by Battistella et al. included six patients treated with intra-arterial BM-MNCs with a time window of 2–3 months from stroke onset [43]. There was no worsening immediately after the procedure or during follow-up period. At the 180-day follow-up evaluation, there was a slight improvement in NIHSS (range −1 to −8 points). Although BM-MNC transplantation was safe in these patients, there is less evidence from the animal studies to suggest that BM-MNCs could be effective in this time window. The same group later published another trial, including 20 patients with moderate to severe middle cerebral artery (MCA) ischemic stroke in a time window of 3–7 days [44], showing no procedure-related adverse events, with 40 % of good clinical outcomes at 6 months.

In 2013, this group (Rosado de Castro et al.) compared IV vs. IA routes in BM-MNC transplantation in 12 stroke patients, demonstrating that with the IV route more cells were trapped in lungs after injection than IA injection. However, they found similar rates of brain homing between both routes [45]. Remarkably, all of the intravenous patients suffered seizures during the follow-up period. Authors hypothesize that the infused cells could modify excitability in the perilesional regions, generating seizures, which should be evaluated further in future clinical trials.

In another trial evaluating the test, feasibility, and safety of autologous BM-MNC infusion in patients with acute ischemic stroke, Savitz et al. [46] included ten patients with a time window of 24–72 h after stroke onset treated with intravenous BM-MNCs. This methodology is supported by a preclinical study in which rats with middle cerebral artery occlusion performed better on neurologic tests with IV mononuclear cells infused up to 72 h, compared with 1 week from stroke onset. There were no study-related severe adverse events. However, of the ten patients included, two of them required hemicraniectomy due to malignant middle cerebral artery infarction after transplantation. In the efficacy analysis, there was a trend toward better outcomes in BM-MNC patients when compared to 79 historical controls who met the NIHSS inclusion criteria.

Our group performed a pilot single-blind (outcomes assessor) phase I/II controlled clinical trial in patients with subacute MCA ischemic stroke [47]. The aim was to assess the safety, feasibility, and clinical effects of autologous intra-arterial BM-MNC transplantation. Twenty patients (ten cases and ten controls) with severe ischemic stroke in the middle cerebral artery territory within 5–9 days from stroke onset were included. The primary outcome was safety and feasibility of the procedure. Secondary outcomes were the improvement in neurological function assessed by modified Rankin scale, Barthel index, and NIHSS.

All were severely disabled at inclusion (mean NIHSS score of 15.6 in BM-MNC group vs. 15.0 in control group, $p=0.82$). BM-MNC transplantation was done at 6.4 ± 1.3 days after stroke onset. A mean 1.59×10^8 BM-MNCs ($\pm1.21\times10^8$) were intra-arterially injected. Rate of infusion through microcatheter was 0.5–1 mL/min, as rates of up to 2 mL/min do not seem to produce cell damage nor the use of heparin or iodine contrast [48].

There were no adverse events related to BM-MNC transplantation. No significant hemodynamic or respiratory changes occurred during the bone marrow harvest or the intra-arterial BM-MNC injection. DWI-MRI did not show new ischemic lesions in the active group after transplantation. During follow-up, two BM-MNC-treated patients had an isolated partial seizure. No deaths or stroke recurrence were observed during the follow-up period, and the 6-month MRI also showed no tumor formation in either group. There were no significant differences in neurological function compared to the control group. At 6 months, a greater nonsignificant proportion of BM-MNC-treated patients had an mRankin (modified Rankin) ≤2 (20 %) than the control group (0 %) ($p=0.47$). No differences were found in the Barthel index ($p=0.80$) or in NIHSS scores compared to the control group ($p=0.43$).

Prasad et al. also reported a trial with 11 stroke patients within 7–30 days from stroke onset [49]. Patients received IV BM-MNC transplantation and were followed up for a year, with no detection of tumor formation or other adverse events related to cell therapy.

The same group published in 2014 the biggest trial to date with BM-MNCs using intravenous route, including 120 patients in a phase II trial [50]. Fifty-eight patients were treated with BM-MNCs and 60 patients were controls. Patients with subacute ischemic stroke between 7 and 30 days were included in the study. A randomization was done in a 1:1 ratio and a single intravenous infusion of autologous BM-MNCs was performed in experimental group with a mean of 280.75 million BM-MNCs at median of 18.5 days after stroke onset. During follow-up, 8.4 % patients died and Kaplan-Meier survival curve showed no differences between both groups. Adverse events and serious adverse events were also comparable between the two arms.

In the efficacy analysis, there were no significant differences between BM-MNC arm and control arm in the Barthel index score (63.1 versus 63.6; $p=0.92$), modified Rankin scale shift analysis ($p=0.53$) or score >3 (47.5 % versus 49.2 %; $p=0.85$), NIHSS score (6.3 versus 7.0; $p=0.53$), or change in infarct volume (-11.1 versus -7.36; $P=0.63$) at day 180. Authors concluded that with the methods and timing used, the intravenous injection of BM-MNCs is safe, but there is no beneficial effect on stroke outcome.

Several other trials are ongoing testing different time windows, doses, and routes, which will give more light about the possible efficacy of BM-MNCs in stroke.

One of the *disadvantages* of BM-MNCs is that the mixture of cells (i.e., myeloid, erythroid, lymphoid, and stem cell populations) makes not possible to perform an allogenic BM-MNC transplantation without immunosuppressive drugs due to rejection. Another issue is the variability in the number of cells obtained after a bone marrow harvest, with a variability in final dose of cells injected when a standardized volume of bone marrow is harvested. In our previous trial, a volume of 50 mL of bone marrow leads to doses as different as $0\cdot33$ and $4\cdot96\times10^6$/kg.

On the other hand, the presence of different populations of cells within the mononuclear fraction of bone marrow could be an *advantage* and seems to be beneficial, as not only stem cells contribute to improved outcomes after stroke [51]. In a recent paper, Yang et al. showed that, in a mouse stroke model, both myeloid cells and stem cell populations are important cell types that reduce inflammation and subsequent infarct maturation. The stem cell subpopulation within BM-MNCs is critical for the therapeutic effect in post-stroke recovery. However, myeloid cells (granulocytes and monocytes) seem to modify also pro-inflammatory cytokines and regulate the microglia decreasing the neurotoxic effect and improving neuron survival rates leading to improve stroke outcomes [52].

10.2.4 Human Multipotent Adult Progenitor Cells (MAPCs®)

Recently, multipotent adult progenitor cells (MAPCs), a subpopulation of stem cells isolated from bone marrow, have been described and characterized. Human MAPCs are multipotent stem cells that have been shown to differentiate into various mesodermal cell types, with a remarkable proliferative capacity in culture. In particular, their vascular potential in vitro and in vivo has been demonstrated which make them an attractive candidate for novel cell-based treatment of ischemic diseases. Moreover MAPCs are also immunoprivileged. In a recent preclinical study comparing MSC and MAPC, the latter compared favorably with hMSC and provides a greater beneficial effect as indicated by the increase in angiogenesis, SVZ cell proliferation, and decreased inflammatory response providing an attractive new source of allogenic source of cells for stroke [53]. With data not yet published, Hess et al. (Table 10.1) have communicated the safety and feasibility of intravenous MAPC therapy in acute stroke patients.

10.3 Timing, Route, and Dose of Bone Marrow Stem Cell Transplantation

10.3.1 Time Window

The *optimal time window* for stem cell therapy is not well known. In the stroke rat model, this time window seems to be wide, even up to 1 month after cerebral infarction, but only rats receiving bone marrow stem cells 7 days after MCA occlusion exhibit decreased ischemic lesion volume [54]. However, some groups have demonstrated that an earlier transplantation results in better neurological recovery, especially when MSC or BM-MNC injection is performed during the first week after stroke or even in the first 72 h [55, 56]. Therefore, it is plausible that an earlier treatment could produce a greater effect on inflammation, apoptosis, and remodeling after stroke. In line with this preclinical evidence, our group described that when

BM-MNCs are administered intra-arterially in subacute MCA stroke patients, they seem to induce changes in serum levels of cytokines and growth factors (i.e., GM-CSF, PDGF-BB, and MMP-2) even 3 months after transplantation, which seem to be associated with better functional outcomes in stroke patients [57].

On the other hand, stem cell transplantation in the acute stroke phase (i.e., within 72 h) could be challenging, as these patients are usually neurologically unstable and prone to deteriorate. Also, this short window needs extensive logistical efforts to perform an autologous bone marrow cell injection in a hospitalized stroke patient. An allogenic transplantation would probably be more feasible in this setting although the usual complications of patients in this early stage of stroke could make more difficult to evaluate safety issues of transplantation.

Although there is no much evidence from preclinical studies to perform a clinical trial with stem cells in the chronic phase of stroke, several trials are treating patients with MSCs or BM-MNCs and stable deficit from chronic strokes [31, 41].

10.3.2 Route of Delivery

Based on animal models of stroke, it is not clear which route of delivery is preferable. Although intravenous (IV) stem cell delivery is increasingly used in clinical trials, IV injection leads to an initial random dispersion of cells throughout the body, and recent data suggest that the majority of the stem cells administered are trapped in filter organs such as the lungs, liver, and spleen, with a therapeutically questionable number of cells reaching the ischemic brain [58]. In contrast, intra-arterial cell delivery provides the opportunity to target the entire ischemic lesion enabling exposure of cells to chemoattractant signals (originating from the lesion). Other routes are being tested such as the intrathecal route [59] or the report of Steinberg et al. [31] using intraparenchymal route with exciting preliminary results, but with some serious adverse events (i.e., subdural hematoma and pneumocephalus).

However, similar to prior animal experiments, clinical trials with IV or IA injection of bone marrow stem cells also have found cells sequestered in the spleen, lung, liver, and kidney [55, 60]. This fact raises the question of whether cells need brain homing to produce the beneficial effects or the cytokine and growth factor secretion is enough to improve stroke outcomes. Although paracrine mechanisms are now the leading hypotheses to explain how cell therapies may enhance stroke recovery [16], it seems critical to expose cells to the ischemic environment to stimulate growth factor production [61].

10.3.3 Cell Dose

A wide range of number of cells has been used for transplantation in animal stroke models and in clinical trials. While in preclinical studies there is strong evidence that a higher dose of cells increases the probability of a good neurological outcome

[23, 55], the optimal number of cells to be transplanted for ischemic stroke is unknown. This raises the question of whether a higher dose of stem cells would produce a greater effect in recovery in stroke patients, but to date clinical data regarding dose is scarce.

Our group [47] found that although no significant correlation between the functional status and the amount of transplanted BM-MNCs was detected, there was a trend toward a better outcome when higher numbers of CD34+ cells were injected. In the three follow-up evaluations, a trend to positive correlation with Barthel index and negative correlations with mRankin scale and NIHSS was found, especially in the Barthel index at 1 month after transplantation ($r=0.57$, $p=0.09$). These data may support the hypothesis that a higher number of cells could lead to better outcomes.

Taguchi et al. [62] evaluated in a clinical trial two different doses of BM-MNCs administered intravenously in stroke patients after 7–10 days of stroke onset (250×10^6 and 340×10^6 cells in the lower and higher dose groups, respectively), and although it was a phase I/IIa clinical trial not designed to test efficacy, authors described a trend toward improved neurological outcomes in those patients receiving the higher dose of bone marrow cells.

On the other side, Prasad et al. [50] published a phase II trial including 120 stroke patients with 58 of them being treated with intravenous injection of BM-MNCs, showing no relationship between cell dose and outcomes.

Also, in a meta-analysis of cell-based therapies for treating stroke patients [63], authors found that stem cell therapy was more effective with higher dose of cells and also when intra-arterial route was used.

In a recent pooling data of two different clinical trials with BM-MNCs [64], a higher dose of autologous BM-MNC was related to better outcome in stroke patients. In this paper, 22 patients were analyzed and intra-arterial route was used in 77.3 % and intravenous in 22.7 % of patients. A higher number of cells injected were associated with better outcomes at 6 months ($p=0.015$). Also, a strong negative correlation was found between cell dose and disability when intravenous patients were excluded from analysis ($r=-0.63$, $p=0.006$), pointing to the hypothesis that the combination of higher number of cells and intra-arterial route could be a key factor to improve neurological outcomes in stroke patients. This pooling data showed that the optimal threshold of transplanted cells is probably around 310×10^6 BM-MNCs in order to obtain good functional outcome with high probability among treated stroke patients. However, further clinical data is needed and dose-finding clinical trials are ongoing in ischemic stroke patients [31, 65].

10.4 Conclusions

As no effective neuroprotective or neurorestorative drug has demonstrated efficacy for ischemic stroke, new therapeutic strategies such as cell therapies to enhance neurological recovery after stroke are urgently needed.

Data from preclinical and clinical studies with stem cells in stroke strengthens the notion that stem cells could increase brain plasticity and improve stroke recovery. Extensive preclinical studies have demonstrated large and favorable effects of different types of bone marrow stem cells in stroke.

Several phase I and II clinical trials have been published to date with bone marrow stem cells that have already demonstrated the feasibility and safety of this therapy in the stroke setting. An increasing number of clinical trials, mainly with bone marrow MSCs and BM-MNCs, are ongoing to further assess the best dose, route, and timing of this therapy and to elucidate the efficacy of cell therapy in stroke.

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Chapter 11
Bone Marrow Stem Cell Delivery Methods, Routes, Time, Efficacy, and Safety

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Abstract Stem cell transplantation offers an exciting new therapeutic avenue for stroke, as many studies have demonstrated favorable results in animal models with various cell types. Several early phase I and II clinical trials are now underway with promising outcomes. However, cell transplantation for stroke is still in its infancy with many issues that need to be addressed in order to achieve full potential as a therapy. Among the major hurdles for a successful clinical translation is determining the optimal conditions of transplantation for stroke. In this chapter, we review the impact of implanted cell number, delivery sites of cells, and transplantation time on the stroke outcome. In addition, we also discuss the efficacy and safety of bone marrow stem cell transplantation for stroke treatment.

Keywords Transplantation • Stroke • Optimal condition • Time • Cell number

11.1 Introduction

Stem cell transplantation offers an exciting new therapeutic avenue for stroke, as many studies have demonstrated favorable results in animal models with various cell types. Several early phase I and II clinical trials are now underway with promising outcomes. Therefore, the potential therapeutic impact of stem cell transplantation on regeneration of damaged brain tissue opens up enormous possibilities. If successful, millions of stroke survivors with disability may benefit. However, cell transplantation for stroke is still in its infancy with many issues that need to be addressed in order to achieve full potential as a therapy. Among the major hurdles

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for a successful clinical translation is determining the optimal conditions of transplantation for stroke. As different groups used different protocols or conditions, the results may not compare well with each other, which makes it difficult to determine the best conditions for stem cell therapy following a stroke. The optimal conditions, including the best cell type, the cell number, the timing of transplantation, the route and site of delivery, and the stroke model, are highly important. All of those conditions still need further study. In the following parts, we will summary recent studies, which are associated with BMSC delivery method, time point, efficacy and safety.

11.2 Delivery Methods and Routes

Bone marrow stem cell (BMSC) transplantation is a promising therapy for some kind of diseases like traumatic spinal cord injury (SCI) and degenerative conditions of the central nervous system (CNS). The number of transplanted cells in the brain depends on the effectiveness of the transplantation. BMSC transplantation has been investigated and explored in animal models to determine its therapeutic effects for disorders such as SCI and brain ischemia [1]. BMSCs produce different trophic factors (e.g., brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF), nerve growth factor (NGF), and hepatocellular growth factor (HGF)) and cytokines. BMSCs do not only activate endogenous restorative responses such as angiogenesis, synaptogenesis, and neurogenesis, they also have a negative effect on the death of the brain cells in the ischemic boundary zone [2]. Prior to the administration of BMSCs, it's required to culture them for a certain amount of time [3]. BMSCs have the protective potentials to repair the tissue [4]. Also BMSCs have the ability to cross the blood–brain barrier (BBB) and migrate into the brain parenchyma [5]. In order to achieve greater efficacy in terms of neuroprotection, BMSCs should be modified and injected intracerebrally [6]. The neuroprotective effects of BMSCs may result from their behavior as small molecular factories [7], although the basic mechanisms are still not comprehensive. Different routes of administration BMSCs exhibit different effects in treating disorders.

11.2.1 Intravenous and Intra-arterial Routes

When BMSCs are intra-arterially administered, larger numbers of the cell are able to reach their target tissues such as the brain parenchyma as opposed to the intravenous (IV) routes. The cells are thus able to confer their neuroprotective functions efficiently through the intra-arterial (IA) routes. Protective factors from the marrow cells provide the neuroprotection after transplantation. Injection of BMSCs into the ipsilateral carotid artery after transient middle cerebral artery occlusion (MCAO) results in many BMSCs in the ischemic hemisphere [1]. Transplantation of BMSCs after brain ischemia results in up to 21 % of the cells in the MCA territory [2]. Administration of BMSCs after brain ischemia decreases infarct volume and increases transplanted cells in the brain when infused in intravenously [8]. Following

transplantations of BMSCs, analysis of brain sections can be performed by fluorescence photography. BMSCs transplanted by IA routes appear to express larger numbers of PKH26-positive cells in an ischemic hemisphere compared to the IV routes. IA transplantation is an effective route in reducing infarct volumes unlike the IV routes. Rotarod score test of an IA-transplanted BMSCs shows improved and higher motor function than the IV-transplanted ones. BMSCs thus improve functional outcome when administered intra-arterially following ischemia [3, 9]. Reports indicate that adverse effects after IA administration of BMSC are often minimal and insignificant in patients with stroke [10–12]. IA routes however require a cerebral angiography which is an invasive procedure with its associated risk factors such as new strokes. The risk is nevertheless very minimal with research indicating the possibility of a new stroke is about 0.14% and other complications is close to 2% [13]. Other notable risk factors following BMSC transplantation intra-arterially involve embolism and occlusion of brain vessels [14]. IV routes thus might be safer than IA routes to some extent. That's not convinced that IA routes seem to be an effective and a superior route than the IV routes. Further research and validation need to be performed to elucidate that one route is more functional than the other [15]. Stem cell delivery to the injured spinal cord has to overcome several arterial branches to maximize efficacy. Hence, a highly selective and technically challenging cannulation is required. One advantage of intravenous stem cell delivery is comparatively the least invasive approach and has been investigated in several studies [16]. After intravenous injection, cellular homing occurs into the pathological CNS tissues. However, IV route is still less efficient when measured with other approaches such as intra-cerebrospinal fluid (CSF). Additional problems associated with intravenous stem cell delivery include reliance on injury-mediated opening of the BBB to allow cell access to the CNS parenchyma (or the need for additional drugs such as lipopolysaccharide to open the BBB) [17]. First-pass effects affect BMSCs and trap them in extra tissues such as the liver and lungs exposing them to longer periods of immunity and reticuloendothelial cells after injection into the bloodstream. Very few to no cells usually present within the injured spinal segments that receive BMSCs intravenously indicates that the effectiveness of cell therapy might not be necessarily related to the number of cells reaching the brain parenchyma [18].

11.2.2 Intrathecal/Lumbar Puncture Routes

Lumbar puncture (LP) is a minimally invasive way of cell and drug delivery, and BMSC may be well suited for LP transplantation because of their responsiveness to signals from the injured CNS [17]. Clinical and theoretical studies show that LP delivery of stem cells is extremely attractive. LP is performed at the L3–4 level, far away from the cervical or thoracic spinal cord, which is the region most commonly effected by SCI. This makes LP delivery of stem cells relatively safe and unlikely to worsen compromised patients as a direct result of the intervention. Stem cells can be injected directly into the lesion site; however, the additional trauma from

intramedullary transplantation can further compromise injured tissue and impede clinical outcomes. A potential and effective alternative is intrathecal transplantation via LP [19]. Additional advantages of LP delivery are related to several factors: (1) they are far more superior to IV route because they are injected directly into the CSF without encountering the BBB; (2) the CSF transports the cells to injured tissues without encountering first effect degradation by the liver or lungs; and (3) because the transplanted cells are delivered away from the hostile environment of the injured tissue, they are given a greater opportunity to survive and migrate to the injured site. Cell transplantation via LP may be relevant for conditions such as multiple sclerosis with widely disseminated lesions making intramedullary transplantation impractical [20]. Neuronal progenitor cells provide neuroprotective functions for injured spinal cord after subacute transplantation directly into the cord [21]. Proliferation of BMSC occurs both in injured and uninjured spinal cord after LP transplantation during the early stages of the transplantation; however, the number of proliferating cells decreases with time. LP-transplanted BMSCs are distributed in the intrathecal space, along the length of the spinal cord, and a few will migrate to the lesion cavity. This implies that BMSC can reach the injured spinal cord using minimally invasive method of LP injection of cells into the lumbar intrathecal space. Prior to transplantation of the cells, extensive incision and disruption of the dura need to be done to allow for transplanted cells to migrate freely into the injured spinal cord [22].

Swelling of the injured cord together with the meningeal reaction causes intrathecally injected cells to be attracted to the cord parenchyma. Collagenous matrix could be implanted to serve as an adhesive substrate for the cells to adhere and attach. The substrate also serves as a barrier for the subarachnoid space to prevent the passage of the intrathecal cells [23].

11.2.3 Cerebral Injections

A major unresolved problem in the context of SCI is the delivery of cells to an already compromised spinal cord without causing further damage. Most investigators have undertaken direct injection into the injured spinal [24]. Although this is acceptable in animal experiments, its extrapolation to humans may be difficult because a major neurosurgical operation will be required. This difficulty in translation will limit clinical trials, at least initially, to patients with complete SCIs in whom further deterioration cannot occur but in whom significant benefit from transplantation therapies is also least likely [25]. Another problem associated with direct parenchymal injections is the likelihood of damaging spared spinal tissues with the injecting needle. It is a well-known principle of neurosurgery that injured tissues do not tolerate operative manipulation as well as normal tissues, because of the presence of edema, altered blood flow, and injury-related cytokines. Finally, direct injection of cells into the parenchyma does not allow suitable delivery of multiple therapeutic doses because of its invasive nature and because injecting cells into multifocal diseases presents many logistical and technical challenges. The direct delivery of stem cells into the CSF has also been explored [26], and intraventricular

injection has been the favored delivery method [27]. This technique, however, is too invasive for clinical applicability, which makes its transplantation challenging. Investigators have demonstrated that neurosphere-derived stem cells delivered into the ventricular CSF can reach the injured tissue in a spinal contusion model [28]. BMSCs are more appropriate because of their evidenced therapeutic effect, their availability, and the possibility of an autologous model in humans. Injection of cells into the lumbar CSF via an indwelling cannula has been shown to be effective for delivering embryonic germ cell derivatives. Considerably more cells will be detected in the injured tissues after both intrathecal and intraventricular delivery. Transplanting cells into the CSF leads to more successful grafting when injection is via an intrathecal or intraventricular rather than intravenous route. The number of cells within the injured spinal cord tissues increased with passage of time. Few cells are present at the early days after transplantation; however, many more cells will be recorded as the time increases after transplantation.

11.3 Effective Times for BMSC Transplantation

Bone marrow stem cell transplantation has over the years been a promising field for the treatment of various disorders like ischemic stroke, hematologic diseases, cardiac disorders like cardiac infarctions, etc. The efficacy of treatment not only depends on the route of cells but also on the cell dose and the time of delivery [29]. For example, de Vasconcelos Dos Santos et al. highlighted the benefit of using BMSCs in the treatment of a thermocoagulation-induced ischemic rat model at different therapeutic windows. They concluded in their experiment that BMSCs might be an efficient treatment protocol for stroke only in the acute/subacute phase of the disease since they were unable to decrease glial scarring significantly [30]. Another example was a recent study which clearly showed that IA administration of 1×10^7 BMSCs immediately after reperfusion is much more effective in delivering BMSCs to the brain than IV administration. In addition, the larger number BMSCs are transplanted in the brain during the early stage of reperfusion, the better protective effect may be presented. The study therefore suggested further understanding into the dose–response influence and therapeutic time window for efficient BMSC delivery to the ischemic site [31].

BMSC transplantation like any other treatment option has an optimum time to which to get adequate outcome. There is therefore the need to optimize treatment by taking advantage of the best time for optimum outcome and reduce disease progress. During ischemia, a series of inflammatory response is initiated which is mediated by many transcription factors of which nuclear factor-kB is a key factor. When hypoxia or ischemia occurs, a cascade of signal transductions is triggered, causing nuclear factor-kB inhibitor IkB phosphorylation degradation and activation of nuclear factor-kB to enter nuclei and stimulate target gene transcription. These processes eventually trigger a positive feedback which leads to an overwhelming inflammatory response. This secondary inflammatory response if not controlled will

accelerate and cause further cerebral ischemia/reperfusion injury. A study performed recently indicated that nuclear factor-kB DNA-binding activity is exponentially enhanced within 6–12 h after ischemia, whereas there is gradual decrease between 24 and 72 h. This indicates that NF-kB translocation occurs in a time-dependent manner after cerebral ischemia [32].

In an experiment to test the hypothesis that IV administration of BMSCs could lead to improvement of functional recovery after MCAO for 45 min in the rat and to determine specific time windows for efficacy. Iihoshi et al. injected rats intravenously with transfected mononuclear cells at 3, 6, 12, 24, and 72 h after MCAO. The ischemic lesion was histologically analyzed at 14 days. It was noted that there was no lesion detected at 3 h transplantation after lesion induction. Lesions were however detected from 6 h post-lesion group and progressively increase at times 12, 24, and 72 h. Infused LacZ(+) bone marrow cells are implanted extensively in and around the ischemic site, with immunohistochemistry studies indicating some amount of differentiation of neuronal and glial cells. Behavioral testing (Morris water maze and treadmill stress test) also indicated improved functional recovery in the transplanted group. These findings further stress the need to intervene as fast as possible and also suggest that IV administration of autologous mononuclear cells from the bone marrow could help improve functional outcome [3]. Other experiments suggest other time windows. Of notable example is the administration of allogeneic human umbilical cord blood MSCs (hUCB-MSC) by LP 3 days after stroke, which was stated to be a valuable method for efficient cell delivery and therapy in stroke model in rats [33, 34].

11.4 Efficacy and Safety of BMSC Transplantation

According to therapy purpose, proper BMSC delivery method should be selected. The delivery efficacy and safety are two critical factors that determine the application of delivery method. When comparing those different delivery methods, every route has some apparently advantages and shortages.

Using IV infusion to deliver BMSC is the simplest and safest method. In animal models, engraftment was demonstrated [35]. However, IV infusion has low cell retention rate; the efficacy is pretty low. Also, BMSC cannot diffuse to specific sites for therapeutic effect. The amount of BMSC arrive the target organ may not efficiently repair the primary injury by differentiation. But, studies demonstrated that recovery improved after BMSC administration should be partially owed to the inflammation milieu improvement by trophic factors and inflammation factors, which are secreted by transplanted BMSC. Comparing to IV infusion, local IA infusion has higher efficacy. The cell distribution after IA infusion of BMSC is much better than IV infusion [36]. IA infusion of BMSC can be applied for heart diseases. IA infusion has some shortage, which may cause microembolism or ischemia during infusion. And sometimes these shortages are fatal. Direct route still is the highest efficacy delivery method, which could deliver a maximum amount of cells to

intended area [37]. Safety would be essential for patients; due to the big invasion, the direct route is limited to some certain situation. LP route is a novel minimally invasive method for delivery of BMSC, which can be applied for many kinds of CNS disease. The important thing is that researches already have demonstrated that BMSC could pass through BBB after LP administration [38, 39]. LP route delivered BMSC able to survive and accumulate and can exhibit the function therapy in CNS disease [22, 40]. The problem is the efficacy of LP still kind of low. Therefore, optimal transplantation technique should be developed to serve maximally safe and efficacy results.

In spite of the restriction of the delivery method, there still have some methods to improving delivery efficacy. Preconditions could improve the survival of BMSC, which including hypoxia and pharmacological treatment. In myocardial infarction model, hypoxia preconditioning can increase the expression of pro-survival and proangiogenic factors including hypoxia-inducible factor 1, angiopoietin-1, vascular endothelial growth factor and its receptor, Flk-1, erythropoietin, Bcl-2, and Bcl-xL. Cell death of hypoxic stem cells and caspase-3 activation in these cells were significantly lower. Transplantation of hypoxic BMSCs after myocardial infarction results in an increase in angiogenesis, as well as enhanced morphologic and functional benefits of stem cell therapy [41]. Indeed, in intracerebral hemorrhage (ICH) model, BMSCs pretreated with hypoxia preconditioning can significantly improve behavioral performance, and increase neurogenesis compared with the vehicle group after ICH [42]. Alternatively, many kinds of pharmacological treatment also could enhance mesenchymal stem cell survival. Trimetazidine (TMZ) preconditioning increases the survival rate of BMSCs through upregulation of HIF1-α in rat myocardial injury model [43]. Hypoxia-inducible factor 1-α (HIF1-α) prolyl hydroxylase inhibitor dimethyloxalylglycine (DMOG) also can upregulate expression of survival and angiogenic factors including HIF1-α, vascular endothelial growth factor, glucose transporter 1, and phospho-Akt, which enhance BMSC survival and therapeutic efficacy after transplantation [44]. Noiseux et al. demonstrated that oxytocin treatment can evoke MSC protection through both intrinsic pathways and secretion of cytoprotective factors [45]. TGF-α stimulates MSC VEGF production in part via a p38 MAPK-dependent mechanism, and preconditioning MSCs with TGF-α could enhance their ability to protect myocardium injury [46]. Tadalafil could increase Bcl2/Bax during the early phase and transcriptional upregulation of PKG-I by STAT3 during the late phase which promotes stem cell protection against ischemic injury [47].

In clinical trials, the efficacy and safety of BMSC transplant for many kinds of diseases have been studied. In stroke patients, the BMSC treatment safety appeared to be safe up to 1 year [48–50]. No significant abnormal EEG/seizures are observed in those patients. Also BMSC transplant treatment in animal stroke model indicated that it has beneficial effects compared to controls [51]. There is another study published by Prasad et al. which showed that intravenous infusion of BMSC doesn't have beneficial effects of treatment on stroke outcome [50]. It is not possible to evaluate efficacy outcome as only one randomized controlled study was available. There still have 15 ongoing clinical trials in phase I or II [52]. After these trials fin-

ish, we can gain more insights into the therapeutic potential of BMSC transplant. In diabetes mellitus, study showed that stem cell transplantation can be a safe and effective approach for therapy [53]. In degenerative diseases of the retina, initial data from early stage clinical trials suggest that short-term safety objectives can be met [54]. However, the question of efficacy will require additional time and testing to be adequately resolved. In spinal cord injury, based on short–medium terms following up, stem cell transplantation appears to be safe and valid in patients and more effective in chronic and complete injury [55]. Nonetheless, prospective, randomized trials in larger cohorts are still needed. In acute myocardial infarction, there is insufficient evidence for a beneficial effect of cell therapy for patients [56]. Further adequately powered trials are needed, and until then the efficacy of this intervention remains unproven.

In summary, base on different disease characters, we can select proper delivery method to increase the safety and efficacy. Preconditions via hypoxia or pharmacological treatments also can improve BMSC survival and enhance the efficacy. Indeed, abundance of researches had demonstrated that BMSCs have beneficial effect on many kinds of disease models. But, for clinical application, the effects of BMSC still need to be confirmed in the following clinical trials. BMSC transplantation therapy is a promising approach for curing so many difficult diseases.

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Chapter 12
Potential Mechanisms of Transplanted Cell-Mediated Recovery After Stroke

Brian Wang, Andrew Hui, and Kunlin Jin

Abstract The underlying mechanisms of transplanted cell-mediated recovery after stroke have received increased attention over the last decade. In this chapter, we review some of the well-known mechanisms in detail and what the more recent findings and relevant clinical trials are suggesting. We also highlight pertinent reports in order to inform the reader of current opportunities and challenges with regard to the following potential mechanisms: (1) functional integration with the existing brain circuitry; (2) neuroprotection through trophic factor secretion; (3) increased formation of new vasculature; (4) structural and functional changes to the brain; and (5) recruitment of endogenous progenitors.

Keywords Stroke • Cell transplantation • Neuroprotection • Angiogenesis • Plasticity

12.1 Introduction

Stroke is the leading cause of adult disability worldwide. Yet, there is only one FDA-approved drug for the treatment of stroke where only 2–4 % of stroke patients can benefit from it due to its short therapeutic window [1]. Therefore, one must look to the post-acute phase of stroke, which has a wider treatment window, and, even more so, therapies that seek to prevent, restore, and repair the damage caused after an episode of stroke. This is where cell transplantation presents itself as an exciting and innovative approach for the treatment of stroke. Several studies have shown

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favorable in vivo results using cells from various sources. Similarly, data from a number of published clinical trials also support the notion of cell transplantation after stroke, and these have been succinctly compiled and reviewed (see [2, 3]). Although the outcomes seem promising, cell transplantation for stroke therapy is still in its stages of infancy that is not without its own unresolved issues. Many cite the following as conditions that need to be met for the successful application of cell transplantation in the clinic (see Chap. 11 for a vivid discussion): optimal timing of cell transplantation, optimal route of administration and site of cell delivery, and optimal number of cells to be transplanted. Of course, these will hardly be rectified any time soon with the myriad of studies differing in their research approach. What might be of help is attempting to understand the probable mechanisms that underlie the phenomenon of transplanted cell-mediated recovery after stroke. Researchers could perhaps obtain insight into the perplexing problems that arise from cell transplantation to further inform them of their future studies.

This chapter endeavors to examine the potential mechanisms in detail and to offer fresh perspectives with regard to this topic. We hope this will not only spark extensive conversations and collaborations to propel the field of cell transplantation forward but will also promote the application of this knowledge to stroke recovery and possibly other diseases of the human body requiring this new lease of life.

12.2 Potential Mechanisms of Transplanted Cell-Mediated Stroke Recovery

Included in the list of potential mechanisms are functional integration with the existing brain circuitry, neuroprotection through trophic factor secretion, increased formation of new vasculature, structural and functional changes to the brain, and recruitment of endogenous progenitors. We detail here recent studies and relevant clinical trials to inform the reader of current opportunities and challenges with regard to mechanisms underlying stroke recovery after cell transplantation.

12.2.1 Functional Integration with the Existing Brain Circuitry

The hope of cell replacement therapy is to restore the brain to its previous normal working condition. When stroke occurs, brain cells in the damaged region die. These are being removed by circulating monocytes that mature into macrophages at the site of injury [4], which leaves an open circuit in the brain that manifests as deficits in the functioning of the body depending on where the stroke developed. It was found that transplanted cells have the potential to form these lost connections by differentiating into what is required to close the circuit again. What is important in functional integration is that the differentiated cells show structural characteristics

analogous to different native neuronal subtypes or glial cells, express synaptic proteins, form synapses with the existing circuitry, and demonstrate synaptic input through electrophysical recordings.

There is evidence that neural stem cells (NSCs) transplanted into the hippocampus of transient global ischemia rats could replace lost circuitry [5]. Although only 1–3 % of the transplanted NSCs survived (out of the 7.5×10^4 being grafted), these NSCs were found to have neurite-like structures in the pyramidal layer of the hippocampus and also express synaptobrevin, suggesting that the transplanted NSCs were able to differentiate into functional neurons and form synapses.

One study that grafted murine embryonic stem cell (mESC)-derived precursors into a rat model of endothelin-1 middle cerebral artery occlusion (MCAO) demonstrated that these mESC-derived precursors were able to differentiate in large numbers into mature neurons with varying neurotransmitter subtypes and supporting glial cells [6]. The group reported the presence of cholinergic, GABAergic, and serotonergic neurons, as well as striatal neurons expressing substance P and DARPP32 at 4 weeks after transplantation using immunohistochemistry. The presynaptic vesicle marker, SV2, was also found to be expressed by the transplanted cells, suggesting their functional maturation. Furthermore, 27.7 % of the transplanted cells exhibited action potentials and voltage-gated Na^+ and K^+ currents, confirming their neuronal phenotype. Unexpectedly, spontaneous excitatory postsynaptic potentials (EPSPs) and excitatory postsynaptic currents (EPSCs) were seen in these neurons between 4 and 7 weeks after grafting, thus establishing their ability to receive synaptic input.

With the advent of human induced pluripotent stem cells (iPSCs), there is a distinct possibility of using patient-specific cells for autologous transplantation [7–9]. However, whether these cells could survive in the long run and differentiate to neurons to promote functional recovery in the ischemic brain are unclear. Lindvall and Kokaia's group was the first to demonstrate that grafting human fibroblast-derived iPSCs is an efficient and safe approach for neuronal replacement in the ischemic brain [7]. They found that functional recovery was seen from just 4 days after grafting the human iPSCs into the striatum of the ischemic rats, while at 5 months after transplantation, the differentiated neurons from the iPSCs were seen to develop axonal projections to the global pallidus and display electrophysiological properties of matured neurons that received synaptic input from the host neurons. This points to the possibility that other mechanisms other than neuronal replacement could be responsible for this early recovery, which makes it more apparent that neuronal replacement may not play a critical role in transplanted cell-mediated recovery in the post-acute phase of stroke. Could it perhaps serve an important function in the chronic stage of stroke that is still yet to be discovered? To truly assess whether or not the transplanted iPSCs or any transplanted cell types are functionally integrated, longer time points are needed to observe further behavioral improvements. Another study showed that grafted iPSC-derived neuronal progenitors that were fated to possess a cortical phenotype in vitro developed into mature and functional cortical neurons, which integrated into the ischemic brains of rats and consequently promoted functional recovery [9]. Interestingly, the groups that were transplanted with cells showed behavioral improvements at 2 months but not at 1 week or 1 month,

once again supporting the notion that another mechanism that alters the microenvironment by the transplanted cells such as immunomodulation [10] could be at play. In fact, it was demonstrated that when neuroinflammation prevailed, grafted cells remained undifferentiated. This could be attributed to the presence of soluble mediators such as BMP-4 and noggin that were secreted by reactive astrocytes, $CD45^+$ cells, and inflamed endothelial cells [11]. Therefore, one can hypothesize that when inflammation-associated cues begin to decrease, grafted cells will then be able to differentiate into functionally mature cell types. Taking it a step further, could this signal a paradigm change with regard to when and how cell transplantation should be performed? Instead of grafting cells early in the stroke recovery phase where inflammation is exceedingly active, studies can perhaps use anti-inflammation strategies to combat the overactive inflammation then proceed with cell transplantation to hasten the process of differentiation and functional integration to achieve behavioral improvement quickly.

A very recent study using what is called multilineage-differentiating stress-enduring (Muse) cells was assessed for fate and function in a rat model of transient MCAO [12]. Muse cells are a distinct stem cell population with self-renewing capabilities and can differentiate into cells that represent all three germ layers (ectoderm, mesoderm, and endoderm) from just a single cell. They are also stress tolerant and were discovered in mesenchymal tissues such as the bone marrow and cultured fibroblasts [13] and have been shown to be non-tumorigenic [13–15]. Muse cells were injected into the ischemic rat's cortex at 2 days after transient MCAO, while the authors observed the behavior and effects of the grafted Muse cells for the following 84 days. Ischemic rats that received Muse cells showed significant improvements in neurological and motor functions at days 70 and 84 compared with control. Despite the fact that infarct volume remained the same, the transplanted Muse cells demonstrated their abilities to increase the survival of the host cells in the peri-infarct area. The grafted Muse cells were able to survive in the ischemic rat brain for a period of 84 days and surprisingly, differentiated with high ratio into neuronal cells (~60%) and with moderate ratio into oligodendrocytes (~25%), while glial fibrillary acidic protein-positive ($GFAP^+$) cells were scarce. Of note, the Muse cells displayed presence of synaptophysin, were able to integrate into the sensory-motor cortex with neurites that extended into the cervical spinal cord, and exhibited the normalization of somatosensory evoked potentials (SEPs).

12.2.2 Neuroprotection Through Trophic Factor Secretion

It has been shown time and again that within the first 2 days of cell transplantation after stroke, there often is a reduction of infarct volume and apoptosis in the peri-infarct tissue [16]. This would suggest a form of protection that is mediated directly or otherwise by the transplanted cells to aid in the recovery process. A plausible mechanism for neuroprotection many studies have found is through transplanted cell-mediated release (and/or stimulation) of endogenous trophic factors such as

brain-derived neurotrophic factor (BDNF), fibroblast growth factor-2 (FGF-2), and vascular endothelial growth factors (VEGFs).

The essentiality of BDNF cannot be understated. It has been shown to sustain physiological processes in the normal adult brain such as learning and memory by modulating dendritic spine morphology and branching [17, 18] and synaptic plasticity [19]. In the poststroke brain, BDNF is involved in protective and reparative processes including neurogenesis, motor learning, and the downregulation of anti-apoptotic proteins [20, 21]. To date, there are only two stroke studies – one in the neonate rat [22] and the other in an adult rat [23] – that have transplanted BDNF-secreting mesenchymal stem cells (BDNF-MSCs) which were genetically modified to overexpress BDNF using an adenovirus construct. van Velthoven et al. [22] delivered rat BDNF-MSCs via the intranasal route to ischemic P10 rats and found significant reductions in infarct size, gray matter loss (both $p<0.01$ when compared with vehicle) and white matter loss ($p<0.05$ vs. vehicle) at 28 days post-MCAO. The effects of BDNF-MSCs, however, did not differ significantly from those rats transplanted with MSCs only. Although BDNF-MSCs promoted a significant reduction in motor deficits at 14 days, the effect was seemingly temporary as there was no noticeable difference at 28 days post-MCAO when compared with the MSCs-only group. Moreover, the BDNF-MSC and MSC groups saw significant increases in SVZ cell proliferation at 28 days post-MCAO, suggesting that there are indeed active endogenous repair processes taking place. Taken together, the data indicate that BDNF-MSCs could confer similar levels of benefits than MSCs alone. This observation can be attributed to the use of a non-integrating adenoviral construct wherein the resulting forced overexpression of BDNF is only temporary as the vector is subsequently removed from the cell. Administering additional BDNF-MSCs at later time points or forcing sustained BDNF secretion may be critical to induce longer-lasting effects. In a different study, Jeong et al. [23] transplanted (at 3 days post-MCAO) a recombinant replication-deficient adenoviral vector with a protein transduction domain that encodes for BDNF to produce a consistently high level of BDNF secretion from human bone marrow-derived MSCs (hBM-MSCs). At 14 days after MCAO, there not only was significant proliferation and differentiation of NSCs and functional recovery but also substantial reduction of apoptotic cell death and infarct volume when comparing the hBM-MSCs with vehicle and MSCs-only groups, suggesting that BDNF could indeed be neuroprotective if its expression could be consistently upregulated for a period of time. Longer-term studies, however, are needed to support the constant upregulation of BDNF as a potential therapeutic for stroke recovery.

Another trophic factor that can be released by transplanted cells after stroke is FGF-2; it is known to regulate stem cell proliferation, differentiation, and migration [24–26] as well as induce neuronal sprouting [27]. Injection of neutralizing antibodies to FGF-2 in newborn rats saw a 60% decrease in DNA synthesis in the hippocampus just after 4 h, suggesting the importance of endogenous FGF-2 in brain development [28]. Further, centrally administering FGF-2 in the adult rat at 1 day after permanent proximal MCAO demonstrated an increase in the rate and degree of functional recovery after 4 weeks when compared with control [29]. Even though

FGF-2 is a very well-studied factor, reports with regard to transplanted cell release of FGF-2 after stroke are scarce. Ikeda et al. [30] demonstrated the effects of functional recovery after MCAO in adult Wistar rats by transplanting MSCs with the FGF-2 gene transferred by a replication-incompetent herpes simplex virus type 1 (HSV-1) artificially fused with an interleukin-2 secretory signal sequence. At 3 and 7 days after MCAO, only FGF-2 production in the FGF-2-MSCs group was substantially increased ($p<0.05$) compared with the sham and MSCs-only groups. Interestingly, the level of FGF-2 was maintained in the ipsilateral hemisphere of the MSCs-only group, while it was shown to decrease over time in the sham group, suggesting that continuous FGF-2 secretion by the transplanted cells is a critical factor in determining the extent of damage caused by an episode of stroke. At day 14 after MCAO, only the FGF-2-MSCs group had a significant reduction in infarct volume ($p<0.05$) compared with the other groups. Furthermore, at 21 days after MCAO, the FGF-2-MSCs group exhibited significant neurological improvement ($p<0.05$) compared with the MSCs-only and sham groups. Although the results indicate that transplantation of FGF-2-MSCs with the HSV-1 vector is an efficacious treatment modality for stroke, it would be interesting to examine the long-term effects of FGF-2-MSCs in aged rats since the aged systemic milieu is vastly different from that of the young. On a different note, one could also consider transplanting NSPCs transduced with FGF-2 as these have been shown to associate with the vasculature, which seems to be critical for NSPCs to remain in a proliferative and undifferentiated state, thereby improving their survivability and integration in an ischemic environment [31].

Last but certainly not the least, the family of VEGFs including VEGF-A, VEGF-B, and placental growth factor is critical for the proper functioning and development of the nervous and circulatory systems [32]. Apart from its involvement in the different phases of vascular development, VEGFs also possess direct trophic and neuroprotective effects. For these reasons, it is not surprising that VEGFs are germane to the topic of stroke recovery. In a rat model of global cerebral ischemia where the hippocampus is the main area of damage, VEGF-A mRNA was demonstrated to be induced in neurons at 12 h after reperfusion, with a peak of 1 day, and in astrocytes at 1 day after reperfusion. Interestingly, the VEGF-A signal seemingly disappeared after 3 days [33]. By the same token, a 3-day intraventricular infusion of VEGF-A starting at 24 h after MCAO in rats revealed behavioral improvements lasting for a minimum of 2 months as well as decreased infarct volume by almost a third at 30 days after MCAO [34, 35], indicating that the timing of VEGF-A administration is crucial to achieving maximum recovery from stroke. With regard to conferring neuroprotection by way of secreting VEGF from transplanted cells, a few reports agree that a possible mechanism is more often than not through vasculogenesis, which is to be expected [36, 37]. However, it is becoming increasingly apparent that VEGF is more than a one-trick pony. One group transplanted neuroepithelial-like stem cells that were derived from human iPSCs expressing VEGF into the striatum and cortex of MCAO mice and rats and discovered that although VEGF was increased in astrocytes and blood vessels in the peri-infarct area, there was no evidence to suggest that the higher VEGF levels cor-

related with VEGF-induced angiogenesis and vascularization [7]. This phenomenon was confirmed in another study they performed [8]. Rather than arguing against a significant role VEGF may or may not have from cell transplantation, this could suggest possible non-angiogenic roles for VEGF. In fact, higher VEGF levels from grafted cells at early time points, e.g., day 14 after stroke, could be involved with promoting endogenous plasticity [38] as well as suppressing inflammation [39].

12.2.3 Increased Formation of New Vasculature

A large indicator of whether tissue can be repaired is whether there is adequate blood flow near the penumbra. Vasculature allows for transmission of metabolites necessary for living tissue, the removal of damaged tissue, and the creation of a new location for existing NSCs to both migrate toward or new NSCs to be generated from [40]. The current treatment model consisting of thrombolytic therapy helps replenish the lack of blood flow during a stroke, but this treatment can sometimes be limiting due to a very small treatment window of 4.5 h. If there was a way to create collateral circulation after a stroke event, this could greatly expand the window for recovery from hours to days [41]. Recent studies provide evidence that transplanted cells can lead to increased angiogenesis, which could serve as a possible repair mechanism for stroke patients. Some of the cell types that lead to neovascularization posttransplantation include bone marrow stem/progenitor cell, adipose tissue-derived stem/progenitor cell, embryonic stem cell-derived cells, and cord blood and peripheral blood stem/progenitor cells [41]. It has been reported that these transplanted cells can lead to the increase of endogenous factors such as vascular endothelial growth factor-A/B (VEGF-A/B), transforming growth factor (TGF), TGF-B, fibroblast growth factor (FGF), stromal cell-derived factor-1 (SDF-1), brain-derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF-1) [42]. As such, the mechanisms that involve these molecules are exciting new avenues that may help serve as possible stroke treatment models. These mechanisms are still being explored, and more data must show whether the new vasculature created via the transplantation of stem cells is physiologic or dysfunctional.

Recent research has been seeking to identify a clear mechanistic model for whether BMSCs promote angiogenesis through factors such as VEGF [43]. In one study, conditioned medium bone marrow mesenchymal stem cells were shown to operate via the PI3K-Akt pathway in response to hypoxia to decrease apoptosis while enhancing angiogenesis [44]. Another study suggested that endothelial nitric oxide synthase (eNOS) plays a critical role in whether transplanted BMSCs can serve to regain physiological function [45]. Mice were infused with BMSCs along with Nω-nitro-L-arginine methyl ester (L-NAME), an eNOS inhibitor. The mice injected with L-NAME were analyzed against control, revealing that the L-NAME group failed to increase angiogenesis and neurogenesis, thus indicating that the eNOS/BDNF pathway is an important regulator in whether angiogenesis will occur

in poststroke states. Moreover, the data is unclear whether this is an effective treatment for all types of patients. One study showed that BMSCs mitigated stroke neurological impairment by promoting angiogenesis, neurogenesis, and synaptogenesis in aged mice, while another study showed that BMSC treatment of type 1 diabetic mice contributed to greater amounts of dysfunctional angiogenesis and increased the risk of cerebral hemorrhaging [46]. Continuing research seems to underscore that the process of angiogenesis does occur; however, there is still much to be learned about the mechanistic causality of BMSC transplantation as it relates to functional vs. dysfunctional angiogenesis. This field is particularly beneficial because BMSCs avoid ethical barriers and is currently in the process of ongoing clinical trials to determine treatment feasibility and safety [47]. Results and analyses are still underway and pending.

In addition to BMSCs, adipose tissue-derived stem cells (ADSCs) are another source of possible transplanted cells that can help increase neovascularization via VEGF and HGF release. These factors help increase endothelial cell survivability, migration, and vessel formation [48]. What makes ADSCs great candidates are that they are abundant and relatively easy to extract without the need of undergoing invasive surgery. There are already clinical trials underway including a phase IIa study that involves the administration of adipose-derived mesenchymal stem cells within 2 weeks of a stroke event. Another clinical study is analyzing the safety of direct injection of human adipose-derived stem cells into the brains of chronic non-hemorrhagic stroke patients (ClinicalTrials.gov Identifier, NCT02813512).

Recent research has provided some clarity regarding the mechanism of action that causes ADSCs to promote neovascularization. One study analyzed TGF-B1 expression in mice and found significantly greater amounts of TGF-B1 in the MCAO + ADSC group when compared to control [49]. This significant increase in expression of TGF-B1 suggests the MCAO + ADSC group was able to create new vessels. In another report, the following proteins were found to be significantly upregulated in rats containing transplanted ADSCs: Notch1, DII4, and Hes1 [50]. This data seems to suggest that ADSCs can increase angiogenesis via the Notch1-DII4 pathway.

Transplanted human cord has also been used as a way to obtain neovascularization via endothelial colony-forming cell (ECFC) separation. In one study, there was evidence of increased CD31 expression along the ischemic border when compared to the control group, which suggests the presence of new endothelial cells [51]. In another study regarding Wharton's jelly mesenchymal stem cells (WJ-MSCs), there were increased levels of the transcription factor FOXF1, which is important in the process of neovascularization [52]. While this evidence is encouraging, the exact mechanism of angiogenesis is still unknown after grafting. Hopefully, more studies will discover a model for how angiogenesis occurs when using human cord blood cells.

As we find more potential sources for transplanted stem cells to promote neovascularization, there needs to be an increased focus on how these sources compare and contrast with one another after transplantation. In at least one study, stem cells derived from the bone marrow were evaluated against Wharton's jelly-derived stem

cells. This was performed by analyzing the aforementioned stem cells via cell migration analysis as well as tube formation assays on human microvascular endothelial cell (HMEC) lines [52]. It was found that WJ-MSCs were able to generate longer tube length when compared to BMSCs and that the HMECs showed migration preference for WJ-MSCs. Increased amounts of chemokine ligand 5 (CXCL5) were also secreted by WJ-MSCs as well. CXCL5 has been shown to be an important mediator in angiogenesis [53], which seems to suggest that WJ-MSCs may have better neovascularization potential than BMSCs. Whether this also directly translates to physiologically better outcomes remains to be seen. Ethical barriers may also play a deciding role in which of these transplanted cell sources become a mainstay in future treatment.

12.2.4 Structural and Functional Changes in the Brain

Thus far we have reviewed a number of ways that the body attempts to recover after an ischemic event through anatomical remodeling including angiogenesis and recovery of neural networks. This section serves to explain other potential areas where brain plasticity can be conducive to treatment after stem cell transplantation.

One of the ways stem cell transplantation can affect plasticity after a stroke is through its effects on microRNA (miRNA) expression. This process is usually performed by the miRNA's role in the regulation of gene expression at both the level of transcription and translation. It has been shown that injected stem cells upregulate endogenous brain cells and stimulate miRNAs through the release of microvesicles. These microvesicles contain modified miRNAs that can exert effects including the stimulation of tissue repair processes such as inflammation, neurogenesis, angiogenesis, and the body's response in a hypoxic environment [54]. An example of an important miRNA expression is miR-133b, which has been shown to increase total neurite length and branch number after being exposed to brain extracts of MCAO-induced mice for 72 h [54]. This process occurs through the transmission of miR-133b from BMSCs to both neurons and astrocytes. Interestingly, the release of microvesicles has a bidirectional effect between stem cells and damaged cells by having both an effect on inducing differentiating BMSCs and repair processes [54]. This bidirectional feedback seems to suggest that a large amount of modulation occurs through the microvesicle release between BMSCs and damaged cells. A similar treatment process model is currently being evaluated to treat brain tumors through the use of BMSC exerting its effects on miRNA. Specifically, BMSCs are being used with miR-146b as a vehicle to help minimize neoplastic proliferation by downregulating VEGF [54, 55]. With such treatments in other types of pathology being treated through similar processes, it is important to consider how other miRNAs may be able to influence areas of concern and to continue research into the exact mechanisms.

In addition to BMSC transplantation, human neural precursor cells have been studied for their effects on plasticity after stroke. In one study, dendrites were Golgi stained and analyzed when comparing human NPC treatment mice against vehicle controls [38]. It was found that those that underwent the human NPC treatment had better outcomes and that the changes in dendritic branching were more proliferative in areas that were closer to the human NPCs. Results also showed that human NPC transplantation increased overall dendritic and axon lengths [38]. A quantitative PCR analysis was performed to reveal which factors were expressed by the human NPCs to clarify what mediators were directly involved. Four of the following factors were involved: VEGF, thrombospondin 1, thrombospondin 2, and secreted protein acidic and rich in cysteine (SPARC). Of these, only SPARC had no effects on dendrites or axons when neutralized. When VEGF, thrombospondin 1, and thrombospondin 2 were neutralized by selective antibodies and compared with controls, it was shown that there were reductions in both dendritic branching and length [38]. While these three factors play a role in stem cell effects on brain plasticity during a stroke, the exact mechanism is still unclear.

12.2.5 Recruitment of Endogenous Progenitors

Another prospective route to treat strokes through the use of transplanted cells includes the recruitment of endogenous progenitor cells. While a healthy individual will have NSPCs primarily located at the SVZ regions of the lateral ventricles and SGZ of the dentate gyrus in the hippocampus, evidence has shown that the body will send signals that cause endogenous NSPCs to migrate toward infarct regions of the brain following ischemic injury. The presence of NSPCs in the penumbra is encouraging because they may offer the possibility of treatment through the reparation of damaged neurons or recruitment of cells that can differentiate into mature neurons [56]. Additionally, this also suggests that there is a pathway where neurogenic NSPCs can flow through to reach infarct regions. For simplicity, we shall refer to this pathway as a biobridge [57]. It has been shown that this biobridge can be stimulated by several chemotactic factors following the transplantation of stem cells such as bone marrow stem cells (BMSCs) [58]. The fact that a biobridge exists after stroke events is not enough. Major obstacles exist, causing most of the migrating NSPCs to die out. Some of these obstacles that affect the biobridge include enzymatic degradation, which causes the loss of structural integrity and deteriorating blood-brain barrier (BBB) resulting in dysfunctional anatomical connections. Even if NSPCs were able to navigate these obstacles, radical oxygen species await newly arriving NSPCs to disrupt their survival capabilities [56].

A good treatment model will circumvent disruptive factors by having a good understanding of the mechanisms leading to migration and survival of NSPCs. While we still have much to learn about the mechanisms, several studies have provided evidence for different factors released by transplanted stem cells that play a role in regulating the migration, proliferation, and differentiation of NSPCs in the

CNS. Here we will discuss research findings related to factors derived from stem cells including stromal cell-derived factor-1 (SDF-1), BDNF, and MMP-9.

Stromal cell-derived factor-1 (SDF-1, also known as CXCL12) released by BMSCs has been shown to cause migration, proliferation, and differentiation of NSPCs as well [58, 59]. SDF-1 binds to chemokine receptor type 4 (CXCR4) and CXCR7 receptors, which are shown to help recruit multiple types of stem/progenitor cells. While CXCR7 receptors are mainly expressed in mature myelin sheath [60], CXCR4 receptors provide the signal to regulate NSPCs, endothelial progenitor cells, hematopoietic stem cells, and endothelial progenitor cells. In addition, CXCR4 has been revealed to play a role in the differentiation of oligodendrocyte progenitor cells (OPCs) leading to re-myelination after stroke-induced white matter damage [60]. This finding was suggested after a study analyzed mice coadministered with AMD3100, an inhibitor of the SDF-1/CXCR4 signaling pathway. Mice treated with AMD3100 had much less myelin sheath integrity than mice that showed expression of CXCR4. Furthermore, the time window of treatment using CXCR4 gene therapy seems to be promising. Research has shown that mice treated an entire week after the stroke event still received therapeutic benefit [60]. Comparatively, current treatment has a short window of only a few hours. Future clinical studies will elucidate whether such treatments are safe and viable options.

Several research studies have shown that SDF-1 can be modulated due to certain factors. However, the exact mechanisms still seem to be lacking. For instance, upregulation of the SDF-1 pathway has been found to be stimulated by decreased oxygen levels and increased amounts of hypoxia-inducible factor-1 (HIF-1) [61]. While it can be suggested then that HIF-1 may increase adhesion, cell proliferation, and survival, it is unclear how and if HIF-1 directly impacts SDF-1. C-kit was found to be another regulator in a study where decreased recruitment of endogenous progenitors was seen after inhibiting c-kit [62]. Additionally, eNOS has been shown to upregulate SDF-1 through a cGMP-dependent process in ischemic mice tissue and plays a role in causing progenitor cells to adhere onto endothelium through ICAM-1- and CXCR4-dependent pathways [62]. Research data does not seem to draw clear indications about whether these factors integrate with one another or have indirect impact with one another.

Another key player in recruiting endogenous progenitors is BDNF, which has been shown to increase axonal growth and synaptic plasticity after stroke events. In one study, BDNF was shown to have a larger presence in the ipsilateral hemisphere of the mouse brain following middle cerebral artery occlusion (MCAO) while showing improvements in functional recovery [45]. This suggests that BDNF-secreting mesenchymal stem cells may be beneficial in treatment. The exact pathways in which BDNF causes migration of stem cells require further research.

MMP-9 is another protein stimulated by stem cell transplantation and has been shown to be of benefit to the biobridge. In one experiment, migration of stem cells correlated with a ninefold increase in MMP-9. Inhibitors of MMP-9 were injected into the mice so that they could be analyzed for neurovascular effects. What was found was that MMP inhibition correlated with dysfunctional neurovascular remodeling as well as significantly decreased amounts of SVZ neurogenic migra-

tion to infarct regions [56]. It can be concluded from this study that MMP plays an important role as to why there are increased amounts of endogenous stem cells in damaged regions.

As mentioned earlier, an arising issue that was discovered in the efficacy of stem cell transplantation has been due to the environment and pathway. Current research is underway to create an encasement scheme to help facilitate the delivery of transplanted stem cells to create a stable environment that allows for safe migration. Some of these schemes attempt to replicate a pathway similar to glial tubes consisting of seeded astrocytes [63]. It has also been shown that these bioscaffolds are able to protect the stem cells from oxidative environments [62]. More experimentation is currently being performed to verify the safety and efficacy of these models. Due to the promising results of research, we are continually learning more about how the transplantation of stem cells will recruit endogenous progenitor cells in the clinical setting ClinicalTrials.gov Identifier: (NCT02448641). Within the next few years, we hope that the clinical trials can provide us with new treatments that will produce a larger treatment window.

12.3 Conclusion

The existing research data and clinical trials continue to show that cell transplantation is an avenue of therapeutic for stroke that is worth investing in. While it is promising to see results, we continue to encourage researchers and scholars to collaborate with one another to take extra steps to provide mechanistic proof in order to extend the field of knowledge not only in breadth but also in depth. It is our hope that the many outstanding questions regarding the individual mechanisms highlighted above would provide the inspiration for challenging existing notions in the decades to come.

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Chapter 13
Bone Marrow Stem Cell-Stimulating Factors and Brain Recovery After Stroke

Li-Ru Zhao and Suning Ping

Abstract Stroke is a major cause of death and long-term neurological disability in adults worldwide. In the United States alone, stroke presents a serious public health problem, and it has created heavy public and personal financial burdens. By contrast to the severity of stroke in public health, the treatment of stroke is very limited. Currently, a clot-dissolving drug (rtPA) is the only treatment available for ischemic stroke. The majority of stroke patients are not able to receive this treatment due to the narrow therapeutic window: 4.5 h after stroke onset. Developing new treatment that fits the majority of stroke patients is a huge challenge for stroke research. Over the past decade, numerous studies have shown the therapeutic potential of stem cell factor (SCF) and granulocyte-colony-stimulating factor (G-CSF), the two essential hematopoietic growth factors, in acute, subacute, and chronic stroke. In this chapter, we have reviewed the biological function of SCF and G-CSF in both the hematopoietic system and central nervous system, summarized the progress of SCF and G-CSF research in adult ischemic stroke in both basic and clinical studies, and discussed the directions for future studies.

Keywords Stroke • SCF • G-CSF • Neuroprotection • Neurorestoration • Cerebral ischemia

Abbreviations

AKT	Serine/threonine kinase or protein kinase B
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BFU-E	Erythroid burst-forming units
BrdU	Bromodeoxyuridine (5-bromo-2′-deoxyuridine)

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CFU-GM	Granulocyte-macrophage colonies
CNS	Central nervous system
ERK	Extracellular signal-regulated kinase
FDA	Food and Drug Administration
G-CSF	Granulocyte-colony-stimulating factor
G-CSFR	Granulocyte-colony-stimulating factor receptor
GFP	Green fluorescent protein
HMGB1	High mobility group protein B1
HPCs	Hematopoietic progenitor cells
HSCs	Hematopoietic stem cells
IL-1	Interleukin-1
i.p.	Intraperitoneal
i.v.	Intravenous
LTP	Long-term potentiation
MEK	Mitogen-activated protein/extracellular signal-regulated kinase
MRI	Magnetic resonance imaging
M-type	Mushroom type
NF-kB	Nuclear factor kappa beta
NMDA	N-Methyl-D-aspartate
NPCs	Neural progenitor cells
NSCs	Neural stem cells
PI3K	Phosphatidylinositol-3 kinase
PSD-95	Postsynaptic density protein 95
rtPA	Recombinant tissue plasminogen activator
s.c.	Subcutaneous
SCF	Stem cell factor
SGZ	Subgranular zone
SHRs	Spontaneously hypertensive rats
STAT3	Signal transducer and activator of transcription 3
SVZ	Subventricular zone
TNF	Tumor necrosis factor
U-type	Uncertain type
YFP	Yellow fluorescent protein

13.1 Introduction

Stroke is the fifth leading cause of death in the United States, and it remains the number one cause of long-term disability in the world. Over the past two decades, major advances have been made in the understanding of the pathophysiology of stroke, while there has not been much progress in the development of stroke treatment [26].

Of all strokes, 87 % are ischemic [61]. Most stroke studies, therefore, target ischemic stroke. Although great efforts have been made in developing treatments for ischemic stroke, only one drug, recombinant tissue plasminogen activator (rtPA) for thrombolysis, has been approved by the Food and Drug Administration (FDA) for treatment of acute ischemic stroke. This therapeutic approach must be initiated within 4.5 h after stroke onset (1995; [31]). Because of the narrow time window for treatment and the potential risk of intracerebral hemorrhage, in fact, only 1–3 % of stroke patients are able to receive this treatment [90]. As a result, more than 97 % of stroke patients lack a specific treatment. Thus, developing new therapeutic strategies to save a patient's life and improve their functional recovery is a major challenge for stroke research.

Stem cell factor (SCF) and granulocyte-colony-stimulating factor (G-CSF) are the essential hematopoietic growth factors that govern the growth, survival, differentiation, and mobilization of bone marrow stem cells [91, 102, 103]. Since 2003, the therapeutic effects of SCF and G-CSF on neuroprotection and neurorestoration in ischemic stroke have been frequently studied. Here we have briefly reviewed current understanding for the biological function of SCF and G-CSF in both the hematopoietic system and central nervous system (CNS), summarized the progress of SCF and G-CSF research in adult ischemic stroke, and discussed the directions for future studies.

13.2 The Origin and Biological Function of the Stem Cell Factor and Granulocyte-Colony-Stimulating Factor

SCF and G-CSF are the key members of the hematopoietic growth factor family and play important roles in regulating hematopoietic stem cell (HSC) proliferation, differentiation, and mobilization [20, 30, 55, 91, 102]. Since the discovery of SCF and G-CSF, great advancements have been made in understanding of the biological function of SCF and G-CSF and in developing pharmaceutical intervention of SCF and G-CSF to treat hematopoietic diseases and to repopulate and mobilize HSCs. Accumulating evidence has shown that SCF and G-CSF are not only crucially involved in the hematopoietic system [9, 53], but they also play a role in the CNS [95].

13.2.1 The Origin and Biological Function of SCF and G-CSF in the Hematopoietic System

SCF exists as a dimeric glycoprotein with a molecular weight of approximately 45 kDa [55]. SCF is classified into two forms: a membrane-bound form and a soluble form. Both the soluble and the transmembrane forms of SCF are biologically active. SCF is produced by the endothelial cells and fibroblasts [50]. Bone marrow

stromal cells also produce SCF, which regulates the hematopoietic cell development in endocrine and paracrine manners. It has been revealed that HSCs and hematopoietic progenitor cells (HPCs) contain SCF mRNA; therefore, the growth and survival of HSCs/HPCs may also be regulated by autocrine synthesis of SCF [9, 67]. The production of SCF is increased by inflammatory stimuli such as interleukin-1 (IL-1) or tumor necrosis factor (TNF) [9].

C-kit has been demonstrated to be the receptor of SCF [6]. C-kit encodes a transmembrane tyrosine protein kinase receptor, and it has been found to express in HSCs/HPCs [2, 54, 89]. SCF/C-kit binding is a key process for SCF to regulate hematopoiesis. SCF acts directly on an enriched population containing HSCs/HPCs to accelerate their entry into the cell cycle [48] and enhance HSC/HPC expansion and survival in vitro [5, 92]. SCF is also crucially involved in the generation of white blood cells and red blood cells. It has been demonstrated that SCF is a key player for stimulating CD34-positive HSCs to form granulocyte-macrophage colonies (CFU-GM) and macroscopic erythroid burst-forming units (BFU-E) [5].

G-CSF is an approximately 24 kDa hydrophobic glycoprotein containing a neuraminic acid moiety, which regulates biological activity of G-CSF [16]. There are two recombinant forms of G-CSF, one is a non-glycosylated form and the other is a glycosylated form. Both of the two forms have similar biological activities and bioavailability when administered subcutaneously or intravenously. Many cells produce G-CSF after appropriate stimulation. Monocytes are the most prominent source of G-CSF [88]. Mesothelial cells, fibroblasts, and endothelial cells have also been found to produce G-CSF [44, 47, 101].

The receptor of G-CSF (CD114; G-CSFR) is a typical cytokine receptor with one transmembrane domain, an intracellular signal transduction domain, and homo-oligomerizes upon ligand binding [16]. G-CSF receptors are expressed on HSCs/HPCs [54] and mature neutrophilic granulocytes, monocytes, and platelets [7, 77]. The function of G-CSF has been demonstrated to play a vital role in directing the commitment of HSCs/HPCs to common myeloid lineage [68].

Numerous studies have determined the effects of the SCF and G-CSF combination (SCF+G-CSF) on hematopoiesis and HSC/HPC mobilization. It has been shown that SCF+G-CSF synergistically enhances the proliferation, differentiation, and survival of HSCs/HPCs [19]. The synergistic effect of SCF+G-CSF in hematopoiesis may be partially regulated by phosphatidylinositol-3 kinase (PI3K) and ERK signaling [19]. SCF+G-CSF has also been demonstrated to have synergistic effects in the mobilization of HSCs/HPCs from the bone marrow to the bloodstream in both laboratory animals and humans [3, 8, 20, 35, 84].

13.2.2 The Biological Function of SCF and G-CSF in the Central Nervous System

In addition to the effects of SCF and G-CSF in the hematopoietic system, increasing evidence shows that SCF and G-CSF also play a role in the CNS. Receptors for SCF and G-CSF have been found to express in the brain [38, 52], particularly in the

neural stem cells/neural progenitor cells (NSCs/NPCs) [39, 64, 73, 96], and in cerebral neurons [73, 96] of adult mice and rats. It has been demonstrated that both SCF and G-CSF can pass through the blood-brain barrier [73, 100]. These findings suggest that hematopoietic growth factors, SCF and G-CSF, may have biological function in the CNS.

The role of SCF and G-CSF in directing NSCs/NPCs to give rise to neurons has been illustrated in both in vitro and in vivo studies. In cultured NSCs/NPCs, G-CSF has been shown to promote differentiation of NSCs/NPCs into neurons in a dose-dependent manner [73]. Infusing SCF into the cerebrolateral ventricle results in increases of newborn neurons in the neurogenic region, the subventricular zone [39]. When adding SCF and G-CSF during the proliferating stage of NSCs/NPCs, SCF in combination with G-CSF (SCF+G-CSF) shows a dual function in directing cell cycle arrest and promoting neuronal fate commitment through the enhancement of neurogenin 1 activity [64]. Together, these studies reveal that SCF and G-CSF are involved in neurogenesis.

Numerous in vitro and in vivo studies have examined the contribution of SCF and G-CSF in neuronal survival and neuronal plasticity. SCF selectively enhanced the survival of cultured embryonic chick dorsal root ganglia neurons [11]. SCF protects cultured neurons from apoptosis through the regulation of MEK/ERK or PI3K/AKT/NF-kB/Bcl-2 pathways [17]. Using cultured cortical neurons, G-CSF has been demonstrated to counteract programmed neuron death via PI3K mediation [73]. SCF acts as a neurotrophic factor supporting neuron survival during the development of the peripheral nervous system [11, 37]. SCF enhances neurite outgrowth in embryonic dorsal root ganglia [36, 37]. SCF+G-CSF synergistically promotes neurite outgrowth and network formation of cultured cortical neurons through PI3K/AKT/NF-kB/BDNF pathway [82]. Mice deficient in either SCF [60] or C-kit [43] display impaired long-term potentiation (LTP) and spatial learning and memory. G-CSF knockout mice show cognitive impairments, LTP reduction, and impairments in neural network formation in the hippocampus [18]. Collectively, these research data suggest that SCF and G-CSF, the two hematopoietic growth factors, act as neurotrophic factors to regulate neuron survival and neural plasticity. These findings provide insights into the potential role of SCF and G-CSF on neuroprotection and neurorestoration in the treatment of stroke.

13.3 The Role of SCF and G-CSF on Neuroprotection and Neurorestoration in Adult Ischemic Stroke

After a stroke, brain tissue that is located in and outside the infarct area undergoes significant changes including primary neuron loss, secondary neuron loss, neuroinflammation, neuron functional reorganization, neural network rewiring, and blood vessel regeneration. Based on the pathological progression and timing poststroke, stroke is classified into three clinical phases: the acute, subacute, and chronic phase. The duration and pathological severity of the three phases vary between individuals and depend on the infarction size, infarct location, cerebrovascular collateral

response, patient's age, and medical comorbidities. Generally, the acute phase of stroke is the first 48 h after stroke, and the subacute phase of stroke is the period between 48 h and 6 weeks or to 3 months poststroke, whereas the chronic phase starts 3–6 months after stroke [33, 42, 63]. The primary neuron loss in the infarct core and penumbra zone [87] occurs during the acute phase of stroke, and the secondary neuron loss outside the infarct area mainly happens in the subacute phase of stroke [33]. In contrast to the pathological features of neuron loss in the acute and subacute phases [63], in the chronic phase, a stroke patient's neurological status becomes relatively stable, and the surviving neurons establish new networks in an effort to take over the function of the dead neurons [10, 13, 85, 95].

As stated above, the pathological features of the three phases of stroke are different. Therefore, the therapeutic strategies for each phase should be specific to the pathological alterations. The challenge of the specific treatment for each phase of stroke, however, is that the precise boundary among the three phases is difficult to identify and distinguish. It is often seen that some targeting molecules, such as NMDA receptor, matrix metalloproteinases, and intracellular mediator HMGB1, may have neuroprotective benefits in the acute phase of stroke but they may also risk negatively influencing the process of brain repair in the later recovery phase [59]. By contrast to the targeting molecules, increasing evidence has shown that administration of SCF and G-CSF in any of the stroke phases appears to be beneficial. Systemic administration of SCF and G-CSF in the acute or subacute phase of experimental stroke displays neuroprotective benefits; when administering during the chronic phase, SCF and G-CSF show neurorestorative effects in enhancing brain recovery.

13.3.1 The Effects of SCF and G-CSF in Acute Stroke

The effects of SCF and G-CSF in acute stroke have been extensively investigated. There is a large body of publications studying the role of SCF and G-CSF in neuroprotection in the phase of acute stroke in both animal models and stroke patients.

13.3.1.1 The Effects of SCF in Acute Stroke

C-kit, the receptor for SCF, has been shown to be increased in the neurogenic regions (the subventricular zone (SVZ) and the subgranular zone (SGZ)) of adult rats 24 h after cerebral ischemia [39]. Intraventricular delivery of SCF for 3 days post-cerebral ischemia [39] or subcutaneous daily injections of SCF during the period of 3 h and 7 days after induction of cortical ischemia [96] result in increases in the number of BrdU-labeled neural progenitor cells in the SVZ in rat models of focal cerebral ischemia. Intraventricular delivery of SCF for 3 days after focal cerebral ischemia enhances neurogenesis in the neurogenic regions [39]. Subcutaneous daily injection of SCF beginning at 3 h and ending 7 days after cerebral cortical

ischemia shows a robust improvement in sensory motor function 1 week posttreatment. The SCF-induced functional improvement lasts more than 10 weeks after treatment, and the infarction size is reduced by SCF treatment [96]. These findings suggest neuroprotective and neuroregenerative effectiveness of SCF treatment in the acute phase of stroke.

13.3.1.2 The Effects of G-CSF in Acute Stroke

In comparison to SCF, G-CSF has been extensively studied in acute stroke.

Several studies have revealed the role of endogenous G-CSF in neuroprotection after stroke. Using rat models of focal cerebral ischemia, Schneider and coworkers [73] reported that both G-CSF and its receptor, G-CSFR, were widely expressed in the neurons throughout the brain. Two and 6 h after focal cerebral ischemia, G-CSF and G-CSFR were strongly increased in the neurons adjacent to the infarct area. In addition to rodents, G-CSFR is also robustly expressed in the peri-infarct neurons of human brain in the acute phase of ischemic stroke [34]. In G-CSF-deficient mice, the infarct volume is increased, and cerebral ischemia-induced neurological deficits are exacerbated as compared to wild-type mice. Systemic injections of G-CSF to the mice lacking of G-CSF before and 2 days after focal cerebral ischemia prevent G-CSF deficiency-induced enlarged infarction size and worsened neurological outcome [76]. These studies suggest that endogenous G-CSF and G-CSFR in neurons play an important role in neuroprotection.

The efficacy of exogenous administration of G-CSF in the acute phase of stroke has been largely examined in rat models [49, 72–74, 78, 80, 96] or mouse models (C57BL mice) of focal cerebral ischemia [27, 46, 79]. G-CSF treatment is initiated at the time points ranging from 30 min to 48 h after induction of ischemia with either single injection [27, 46, 73, 74, 79] or daily injections up to 10 days post-ischemia [49, 72, 73, 78, 80, 96]. Systemic administration of G-CSF (s.c., i.v., or i.p.) with treatment dosages of 50 μg/kg or 60 μg/kg shows beneficial effects in reducing infarction size and ameliorating neurological deficits [27, 46, 49, 72–74, 78–80, 96]. These research findings indicate that exogenous administration of G-CSF in the acute phase of stroke protects neurons from ischemic injury.

Understanding the mechanism underlying G-CSF treatment-induced neuroprotection in the acute phase of cerebral ischemia remains incomplete. Accumulating evidence shows that G-CSF treatment in the acute phase of focal cerebral ischemia reduces the disruption of the blood-brain barrier [49], reduces brain edema [28], decreases glutamate release in the infarcted striatum [32], suppresses pro-inflammatory cytokines and inflammatory mediators in the peri-ischemic areas [28, 74], and inhibits peripheral inflammatory cell infiltration to the ischemic hemisphere [49]. Anti-apoptosis may be involved in G-CSF-induced neuroprotection in acute ischemic stroke. Activation of STAT3/Bcl2 signaling, which plays a role in inhibiting apoptosis, is increased in the ipsilesional hemisphere by G-CSF treatment in the acute phase of cerebral ischemia [46, 70, 80]. G-CSF treatment in acute ischemic stroke reduces the number of cleaved caspase-3 expressing neurons in the

injured cerebral cortex [80]. In addition, BrdU-labeled proliferating cells are increased in the ipsilesional hemisphere [78] or peri-infarct areas [96] after G-CSF treatment in acute cerebral ischemia. Neurogenesis and angiogenesis in the ipsilesional hemisphere are also enhanced by G-CSF treatment in acute ischemic stroke [49, 75]. However, these studies demonstrate the correlation between G-CSF-induced neuroprotection and G-CSF treatment-related cellular and molecular events such as anti-inflammation, anti-apoptosis, and pro-angiogenesis. The causal link mechanism of G-CSF-induced neuroprotection remains to be determined.

Controversial results have been reported regarding the neuroprotective role of G-CSF in acute stroke. Using a mouse model of permanent occlusion of the middle cerebral artery in CB-17 mice, Taguchi and colleagues [83] reported a negative result of G-CSF in acute ischemic stroke. Subcutaneous (s.c.) delivery of G-CSF (0.5, 5, 50, or 250 μg/kg) beginning at 1 h or 24 h poststroke and continuing up to 3 days or 7 days resulted in no change in infarction size at 3 days poststroke but increases in brain atrophy at 35 days poststroke in all tested doses except for the lowest one (0.5 μg/kg). The G-CSF treatment also impaired neurobehavioral function and increased infiltration of inflammatory cells (CD11b expressing cells and F4/80 positive cells) into the peri-infarct area. This negative study raises a caution flag on the neuroprotective effectiveness of G-CSF in acute stroke.

Two studies of meta-analysis have assessed the effects of G-CSF on acute stroke using animal models of focal cerebral ischemia. These studies have revealed that (1) G-CSF treatment initiating within 6 h or later than 6 h post-ischemic induction reduces infarction size and enhances functional recovery and the effectiveness of treatment within 6 h poststroke shows a dose-dependent manner [56] and (2) G-CSF treatment reduces motor impairments and death, while G-CSF reduces infarction size in transient but not in permanent models of focal ischemic stroke [22].

Clinical trials of G-CSF intervention for acute stroke have been carried out. In a phase IIa study, intravenous delivery of G-CSF to acute ischemic patients at doses of 30–180 μg/kg over the course of 3 days was reported to be safe and well tolerated [71]. In a small patient-sized phase I clinical trial, G-CSF was also proven safe and well tolerated in acute ischemic stroke patients, and G-CSF intervention showed improvement of neurological function [58]. However, the positive results do not display in a large phase IIb trial [69]. In this multinational, multicenter, randomized, and placebo-controlled trial (NCT00927836), G-CSF (135 μg/kg, i.v. over 72 h) was administered within 9 h post-ischemic stroke to the patients with an infarct location in the middle cerebral artery territory. However, G-CSF intervention failed to reduce the infarct size and improve functional outcome.

13.3.1.3 The Effects of SCF+G-CSF in Acute Stroke

Using a cerebral cortical ischemia model of stroke in spontaneously hypertensive rats (SHRs), in addition to SCF or G-CSF alone treatment [96], we have determined the therapeutic effects of SCF+G-CSF in acute stroke [96]. In this study, SCF+G-CSF was subcutaneously injected for 7 days beginning at 3 h post-ischemia. Similar

to treatments of SCF or G-CSF alone, the combined SCF+G-CSF treatment led to reduction of infarction size. The BrdU-labeled cells in the peri-infarct area were also increased by SCF+G-CSF treatment. In contrast to the robust effects of SCF alone treatment in somatosensory and motor function recovery at 1 week posttreatment, SCF+G-CSF-treated rats did not show functional improvement at this early time point. However, the SCF+G-CSF-treated rats displayed a delayed but long-lasting improvement of somatosensory and motor function, which was detected at 4, 7 and 10 weeks after treatment. Toth and coworkers [86] examined the efficacy of SCF+G-CSF treatment in acute stroke using a cerebral cortical ischemia model of stroke in C57BL mice. SCF+G-CSF treatment, which initiated at immediately after ischemia followed by daily injections (i.p.) for 5 days, reduced infarct volume, promoted homing of BrdU/GFP co-expressing bone marrow-derived progenitor cells into the ischemic brain, increased $CD31^{+}/GFP^{+}$ endothelial cells in the ipsilesional hemisphere, and increased angiogenesis. Using the same mouse model of ischemic stroke as stated above [86], SCF+G-CSF was administered daily (s.c.) during the period of 1–10 days after induction of cerebral ischemia. In addition to reducing infarction size, SCF+G-CSF treatment increased bone marrow-derived neurons in the peri-infarct area and improved spatial learning and memory [45]. These studies shed light on the involvement of bone marrow-derived progenitor cells in SCF+G-CSF-induced beneficial effects in acute ischemic stroke.

13.3.2 The Effects of SCF and G-CSF in Subacute Stroke

There are even fewer studies targeting the therapeutic effects of SCF and G-CSF in subacute stroke than the studies of acute stroke. Using a rat model of transient focal ischemia, Lee and coworkers [49] determined the efficacy of G-CSF in subacute stroke. G-CSF was injected daily (i.p.) for 3 days beginning at 4 or 7 days post-ischemia. G-CSF treatment initiated at 4 days post-ischemia showed better functional improvement and greater reduction in hemispheric atrophy when compared to the treatment that was initiated at 7 days post-ischemia. Using a mouse (C57BL) model of cerebral cortical ischemia, Kawada and colleagues [45] examined the effects of SCF+G-CSF in both acute stroke (treatment during 1–10 days post-ischemia) and subacute stroke (treatment during 11–20 days post-ischemia). They observed that the treatment in both acute and subacute stroke reduced infarction size; however, SCF+G-CSF treatment in the subacute phase of cerebral ischemia showed better improvement in functional recovery and greater increases in bone marrow-derived neurons in the ipsi-infarct hemisphere and in NPC proliferation in the neurogenic region (the SVZ). In addition, they also found that SCF+G-CSF displayed a synergistic effect in promoting proliferation of NPCs in the SVZ as compared to SCF or G-CSF alone treatment. A study from the same group of Kawada showed that SCF+G-CSF treatment in the subacute phase of cerebral ischemia upregulated IL-10, an anti-inflammatory cytokine, on a much greater scale than the treatment in the acute phase [57]. Overall, it appears that the optimal timing

for treatment is quite different between the treatment of G-CSF alone and SCF+G-CSF combination treatment: for G-CSF, the earlier the better, while for SCF+G-CSF, the later the better. The mechanism underlying this timing sensitivity for G-CSF or SCF+G-CSF treatment remains largely unknown. It provides an insightful notion, however, in directing future therapeutic studies for stroke: treatment efficacy depends on the timing of the intervention.

A phase IIb single-center, randomized, and placebo-controlled clinical trial for assessing the safety of G-CSF in subacute stroke has been completed. In this clinical study, G-CSF (10 μg/kg) or placebo was given subcutaneously for 5 days to 60 stroke patients. The treatment was initiated 3–30 days after stroke. G-CSF treatment showed a trend toward the reduction of the infarct volume in magnetic resonance imaging (MRI) results but did not cause significant adverse effects as compared to placebo controls, suggesting that G-CSF is safe when administered in the subacute phase of stroke [23].

13.3.3 The Effects of SCF and G-CSF in Chronic Stroke

Over the past decade, our research team has demonstrated the efficacy and possible mechanisms of SCF and G-CSF on stroke recovery in the chronic phase of stroke using rodent models of cerebral cortical ischemia.

SCF and G-CSF intervention in chronic stroke has been tested and validated to be effective when administered 3.5–6 months after cerebral cortical ischemia in both SHRs, C57BL mice, or transgenic mice with C57BL genetic background [13, 14, 51, 65, 66, 99].

First, we determined the efficacy of SCF and G-CSF treatment on stroke recovery in the chronic phase using a cerebral cortical ischemia model in SHRs.

The rationale for using SHRs is that hypertension is the most important risk factor for stroke in humans [41]. Chronic hypertension causes extensive changes in the cerebrovascular bed [4, 40]. Occlusion of the middle cerebral artery distal to the striatal branch and/or of the ipsilateral common carotid artery in SHRs leads to a more consistent and larger infarction in the cortex than in normotensive rats because of inadequate blood flow through collateral vessels [4, 12, 21, 29, 81, 94, 96–99]. In addition to the consistent infarction, this model also induces permanent deficits in somatosensorimotor function that last up to the chronic phase of stroke. Further, this model has no problem for long-term survival [62, 81, 94, 96–99]. Using the cortical ischemia model in SHRs, SCF (200 μg/kg), G-CSF (50 μg/kg), or SCF+G-CSF was subcutaneously administered for 7 days beginning at 3.5 months post-ischemic stroke. We found that only the SCF+G-CSF combination treatment led to a stable and long-term (17 weeks) improvement in somatosensory motor function. SCF alone treatment resulted in functional improvement but the improvement did not present as stable as the SCF+G-CSF combination treatment. G-CSF alone treatment did not show functional benefits. In addition, field-evoked potentials further validated the neurobehavioral findings and revealed that a normal pattern of somatosen-

sory pathways was reestablished by SCF+G-CSF treatment. In addition to the functional improvement, the infarct cavity was reduced in SCF+G-CSF-treated SHRs [99], suggesting that neural regeneration may be involved in brain repair by SCF+G-CSF treatment in the chronic phase. This study provides first evidence that functional restoration in the chronic phase of stroke is possible through the SCF+G-CSF combination treatment.

We have also assessed the safety and effectiveness of the SCF+G-CSF combination treatment on stroke recovery in the chronic phase using aged animals [65], because stroke has the highest incidence in those over the age of 60 [61]. Aged male SHRs (11–13 months) and C57BL mice (16–18 months) were subjected to focal cerebral cortical ischemia. These ages of SHRs and C57BL mice are equivalent to 61–72 years in humans based on their differences in average lifespan [65]. Six dosages were examined examined in the chronic phase of ischemic stroke in the aged SHRs and C57BL mice: (1) SCF+G-CSF at 200 μg/kg for SCF and 50 μg/kg for G-CSF, (2) SCF+G-CSF at 100 μg/kg for SCF and 25 μg/kg for G-CSF, (3) SCF+G-CSF at 50 μg/kg for SCF and 25 μg/kg for G-CSF, (4) SCF+G-CSF at 20 μg/kg for SCF and 10 μg/kg for G-CSF, (5) SCF+G-CSF at 10 μg/kg for SCF and 5 μg/kg for G-CSF, and (6) SCF+G-CSF at 5 μg/kg for SCF and 2.5 μg/kg for G-CSF. Subcutaneous injections of SCF+G-CSF were given for 5 days beginning at 3–4 months after induction of cerebral ischemia. We observed that all six tested dosages did not cause either acute or chronic toxicity to the livers and kidneys, demonstrating that SCF+G-CSF treatment for chronic stroke is safe for the aged population. When determining the effects of SCF+G-CSF in mobilizing bone marrow stem cells into the blood, the three higher dosages of SCF+G-CSF showed significant elevation of C-kit-expressing stem cells in the blood. In a somatosensory motor testing (limb placement test), two higher dosages of SCF+G-CSF (100/25 μg/kg, and 50/25 μg/kg) led to stable and long-term functional improvement. The intermediate dose of SCF+G-CSF (20/10 μg/kg) showed a short-term improvement, whereas the two lower dosages did not improve somatosensory motor function in the chronic phase of stroke in aged SHRs. These findings suggest that the SCF+G-CSF combination treatment for chronic stroke recovery is a safe and effective approach for the aged population. SCF+G-CSF combination treatment in chronic stroke mobilizes bone marrow stem cells and improves functional recovery in a dose-dependent manner.

We have carried out several mechanistic studies to understand how SCF+G-CSF combination treatment repairs a stroke-damaged brain in the chronic phase. Using a bone marrow transplantation approach to track bone marrow-derived cells, our study revealed that bone marrow-derived endothelial cells and bone marrow-derived neurons were involved in SCF+G-CSF-enhanced angiogenesis and neurogenesis in the brain of chronic stroke [66]. To determine the effects of SCF+G-CSF in regulating dynamics of synaptic circuits in the chronic phase of experimental stroke, we used two-photon microscopy to scan the brain area adjacent to the infarct cavity before and after SCF+G-CSF treatment in aged Thy-1-YFPH mice (C57BL background) [13] (Fig. 13.1). The Thy-1-YFPH mice express yellow fluorescent protein (YFP) only in the layer V pyramidal neurons [24]. Before treatment, the number of

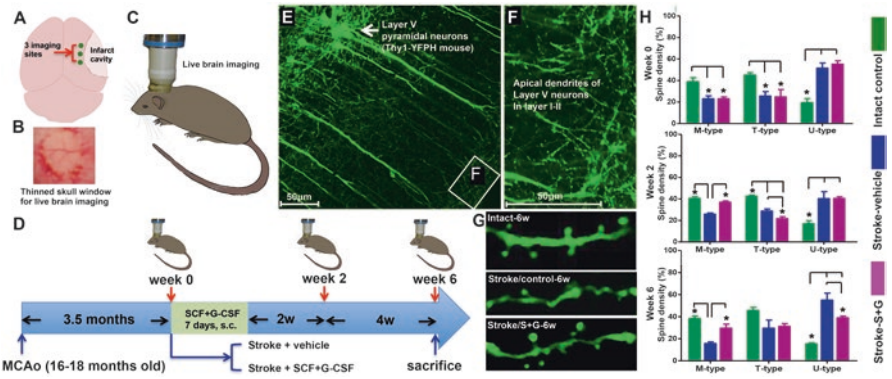


Fig. 13.1 Rebuilding synaptic circuits in the peri-infarct cavity cortex are enhanced by SCF+G-CSF treatment in the chronic phase of experimental stroke in aged mice. (**a**) Schematic diagram showing the three imaging sites adjacent to the infarct cavity. (**b**) Schematic diagram showing a thinned skull window that was prepared for live brain imaging. (**c**) Schematic diagram of the live brain imaging. (**d**) Schematic diagram of experimental design. (**e**) Layer V pyramidal neurons in the cortex of Thy-1-YFPH mice. Note that *yellow* fluorescent protein (YFP) is only expressed in the layer V pyramidal neurons. Boxed area is the layer I–II of cortex enlarged in the panel F. (**f**) The apical dendrites and dendritic spines of the layer V pyramidal neurons distributing in the layer I–II of cortex of Thy-1-YFPH mice. (**g**) Representative live brain images of the apical dendrites and dendritic spines in the brains of intact controls, stroke-vehicle controls, and stroke-SCF+G-CSF-treated mice at 6 weeks posttreatment. Note that the majority of dendritic spines in the cortex of intact controls are mushroom type of spines (M-type), while the majority of dendritic spines in the cortex of stroke-vehicle controls are uncertain type of spines (U-type). The mushroom type of spines appears to be increased in the cortex of stroke-SCF+G-CSF-treated mice. (**h**) Dynamics of dendritic spines before and after treatment. Note that M-type spines and thin-type of spines (T-type) are reduced in the two stroke groups, while the U-type spines are increased in the two stroke groups before treatment. These findings suggest that synaptic degeneration or reduced synaptic circuits occurs in the peri-infarct cavity cortex in the chronic phase of experimental stroke. Two weeks after treatment, stroke-SCF+G-CSF-treated mice show increases in M-type spines and decreases in T-type spines, suggesting the reestablishment of synaptic circuits in the peri-infarct cavity cortex. Six weeks after treatment, M-type spines are increased, and the U-type spines are reduced in the stroke mice treated with SCF+G-CSF. These data indicate that SCF+G-CSF treatment rebuilds synaptic circuits in the peri-infarct cavity cortex. This figure summarizes the results of the study published elsewhere [13]

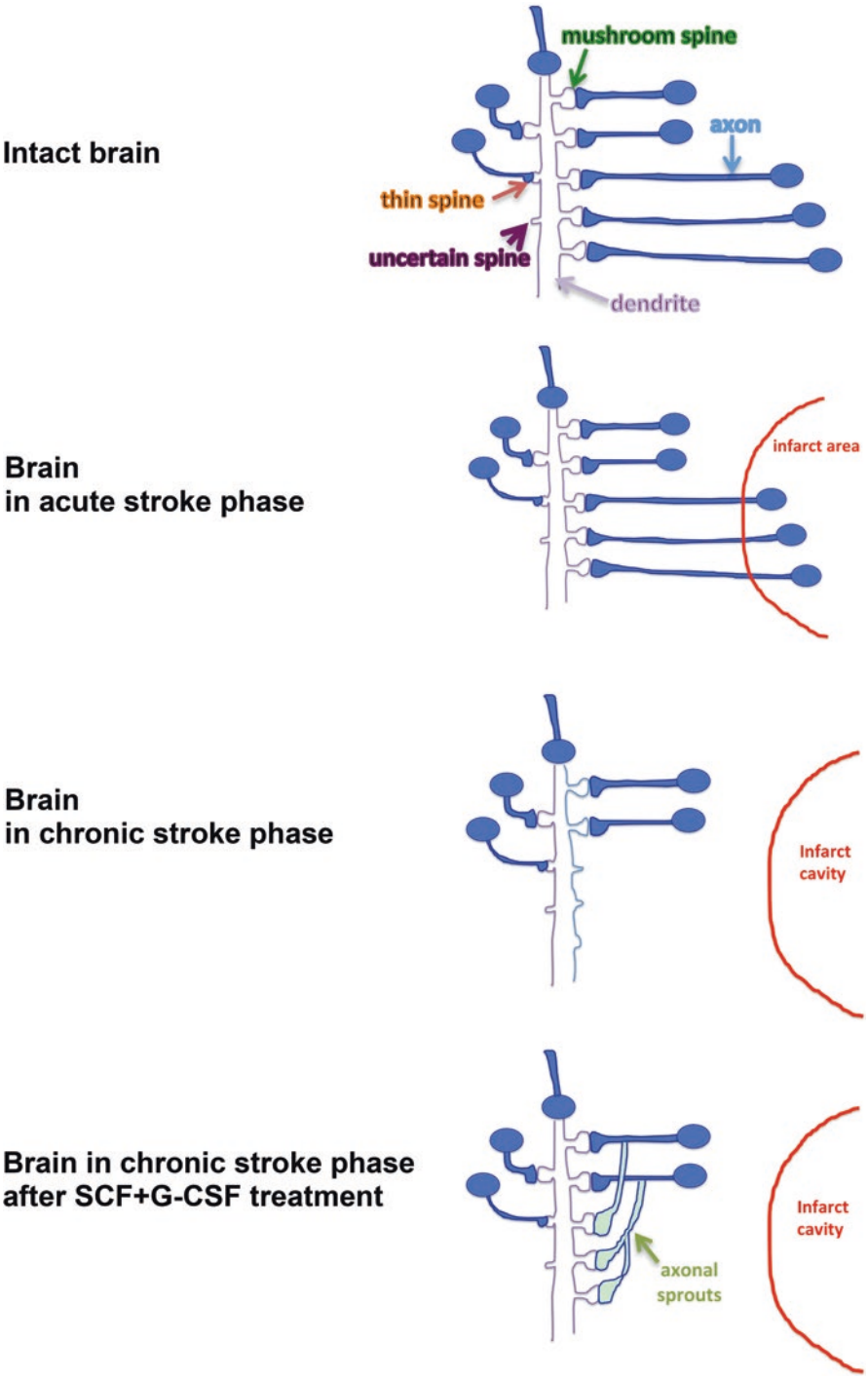
mushroom-type (M-type) spines in the layer V pyramidal neurons was reduced, and the uncertain-type (U-type) spines, which cannot build synapses with other neurons, were increased in the stroke mice. This observation suggests that reduced synaptic circuits occur in the peri-infarct cavity cortex in chronic stroke brain. Six weeks after SCF+G-CSF treatment, however, the M-type spines were significantly increased, and the U-type spines were significantly reduced in the layer V pyramidal neurons adjacent to the infarct cavity. In addition to the two-photon live brain imaging, immunohistochemistry data showed significant increases of postsynaptic density protein 95 (PSD-95) puncta and dendritic branches in the peri-infarct cavity cortex 6 weeks after SCF+G-CSF treatment. These findings suggest that SCF+G-CSF treatment in the chronic phase of stroke enhances synaptic network regenera-

tion in the peri-infarct cavity cortex (Fig. 13.2). This study advances the current knowledge of stroke recovery: an aged brain damaged by ischemic stroke is reparable by a pharmaceutical approach, SCF+G-CSF.

Our follow-up studies have clarified that neural network rewiring in the peri-infarct cavity cortex is required for SCF+G-CSF-enhanced functional improvement in the chronic phase of experimental stroke. In an in vitro study, we have demonstrated that SCF+G-CSF synergistically enhances neurite outgrowth and neural network formation through NF-kB mediation [82]. In an in vivo study [14], we sought to use an approach for blocking the NF-kB-mediated neural network rewiring to determine the causal link between the SCF+G-CSF-promoted neural network regeneration and SCF+G-CSF-enhanced functional improvement in the chronic phase of experimental stroke. In this study, NF-kB inhibitor was infused into the lateral ventricle in the contralesional cortex before and during the 7-day subcutaneous injections of SCF+G-CSF. Motor function was evaluated before treatment as well as 2 and 6 weeks after treatment. Our data revealed that SCF+G-CSF treatment in the chronic phase of stroke increased axonal sprouting, synaptogenesis, and angiogenesis specifically in the peri-infarct cavity cortex but not in the contralesional cortex. NF-kB inhibitor completely abolished the SCF+G-CSF-increased axonal sprouting, synaptogenesis and angiogenesis in the peri-infarct cavity cortex 10 weeks after treatment. In addition, the SCF+G-CSF-improved motor function at 2 and 6 weeks posttreatment was also eliminated by NF-kB inhibitor. This study demonstrates a key role of neural network rewiring in the peri-infarct cavity cortex in the SCF+G-CSF-enhanced motor function recovery in the chronic phase of stroke.

To further identify whether the SCF+G-CSF-enhanced synaptic network rewiring and the SCF+G-CSF-enhanced motor function recovery occurred simultaneously, we carried out an independent study. In this study [15], using a combination approach through live brain imaging, whole brain imaging, molecular manipulation, synaptic and vascular assessments, and motor function examination, we further validated our findings that the SCF+G-CSF-enhanced motor function recovery in the chronic phase of stroke was linked to neural network rewiring in the peri-infarct cavity cortex. Thy-1-YFPH mice were also used for this study. SCF+G-CSF treatment was initiated at 6 months post-experimental stroke. Similar to the earlier study [14], infusion of NF-kB inhibitor was used for blocking the SCF+G-CSF-enhanced neural network rewiring in the peri-infarct cavity cortex.

A previous study reported that motor activity in a Rota-Rod could modify dendritic spine formation [93]. To prevent altering dendritic spines by repeated motor function tests with a Rota-Rod, the chronic stroke mice without behavioral tests were used for live brain imaging and whole brain imaging to identify SCF+G-CSF per se induced remodeling of synaptic circuits in the cortex adjacent to the infarct cavity and/or in the contralesional cortex and to determine whether the synaptic circuit rewiring in the peri-infarct cavity cortex simultaneously happens when motor function is improved by SCF+G-CSF treatment. Our findings showed that SCF+G-CSF treatment at 6 months poststroke improved motor function recovery. SCF+G-


Intact brain
mushroom spine
axon
thin spine
uncertain spine
dendrite
Brain
in acute stroke phase
infarct area
Brain
in chronic stroke phase
Infarct
cavity
Brain in chronic stroke phase
after SCF+G-CSF treatment
Infarct
cavity
axonal
sprouts

CSF promoted mushroom spine formation, enlarged postsynaptic membrane size, and increased postsynaptic PSD-95 accumulation and blood vessel density in the peri-infarct cavity cortex but not in the contralesional cortex. When two-photon live brain imaging showed SCF+G-CSF-enhanced synaptic circuit regeneration in the peri-infarct cavity cortex 2 and 6 weeks posttreatment, motor functional improvement was also seen in the SCF+G-CSF-treated mice 2 and 6 weeks posttreatment.

Once the SCF+G-CSF-increased synaptic network regeneration in the peri-infarct cavity cortex was blocked by NF-kB inhibitor, the SCF+G-CSF-improved motor function was also eliminated. This study has further confirmed that the enhanced neural network formation in the peri-infarct cavity cortex via NF-kB regulation is crucially involved in the SCF+G-CSF-improved motor function in chronic stroke.

A double-blinded, randomized, and placebo-controlled clinical trial for examining the safety and efficacy of G-CSF in chronic stroke has been conducted. Forty-one ischemic stroke patients (>4 months after stroke) were included in this trial. G-CSF (10 μg/kg, s.c.) was given for 10 days. The results showed that the G-CSF treatment was safe to the chronic stroke patients, whereas the improved functional outcome was not seen in G-CSF-treated patients. Authors discussed including more patients in future studies to increase the power of statistical analysis [25].

13.4 Concluding Remarks

Unlike rtPA therapy that has a limited therapeutic window within 4.5 h after ischemic stroke onset, SCF and G-CSF appear to have broad therapeutic potential for acute, subacute, and chronic stroke according to the basic studies using animal models of focal ischemic stroke. The majority of these studies used one-dose and one-time treatment, and some of the studies examined different dosages and treatment time points. Based on the findings, the optimal time for G-CSF treatment appears to be the acute phase or earlier subacute phase of stroke; for SCF, the best treatment time may be the acute phase of stroke; and for SCF+G-CSF combination treatment, the optimal treatment time appears to be the later stage – the subacute and chronic

Fig. 13.2 Schematic diagram of synaptic networks in different conditions. In intact brain, the majority of dendritic spines are the mushroom-type spines. Thin-type spines are the flexible spines that either grow into large mushroom spines or shrink/disappear in response to microenvironment changes. The uncertain-type spines are the spines under degeneration, and this type of spine cannot form synaptic connections with other neurons. During the acute phase of stroke, neurons in the infarct area die due to lack of blood supply. As a result, the post-synapses of the dead neurons undergo degeneration (mushroom spines shrink to uncertain-type spines). SCF+G-CSF treatment in the chronic phase of experimental stroke promotes axonal sprouting and dendritic branching and enhances mushroom spine formation and synaptogenesis. Thus, the SCF+G-CSF treatment enhances rebuilding of synaptic circuits and neural networks in the peri-infarct cavity cortex in the chronic phase of experimental stroke. The schematic diagram shown in this figure is based on our previous studies published elsewhere ([13, 14], 2016)

phases of experimental stroke. However, it remains largely unknown why the treatments of SCF and G-CSF alone or combination treatment have different optimal timings and how SCF and G-CSF alone or combination treatment protects neurons from ischemic injury or restores/repairs neuron function after stroke.

Before moving to clinical trials, several crucial questions need to be addressed: Do we use the most clinically relevant animal models of stroke to determine the therapeutic effects? Do we clarify the precise pathological features for acute, subacute, and chronic phases of stroke? Do we demonstrate the optimal intervention timing, dosage, and delivery route for SCF and G-CSF alone or combination treatment? Do we validate the research findings using different animal models especially in nonhuman primates?

Although clinical trials have proven the safety of using G-CSF in treating acute, subacute, and chronic stroke patients, the efficacy of G-CSF in functional recovery has not yet been demonstrated positively. It is worth noting that keeping the infarct type/size and treatment time point uniform is relatively easier in basic science research using animal models than clinical studies using stroke patients. The variation in infarction size and location, cerebrovascular collateral response, patient's age, sex, race, and medical comorbidities as well as differences in intervention timing may cause robust increases in standard deviation of research data. As a result, significant increases of sample sizes (number of stroke patients) are required for reaching the levels of statistical difference in clinical trials.

Overall, SCF and G-CSF research brings new hope for developing a new treatment for stroke as these hematopoietic growth factors show therapeutic potential for acute, subacute, and chronic stroke. Many open questions, however, need to be addressed in the future for both basic research and clinical trials.

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Chapter 14
Imaging and Tracking Transplanted Bone Marrow Stem Cells After Stroke

Hongbin Han, Weifang Zhang, Lan Yuan, Junhao Yan, Wei Wang, Chunyan Shi, and Yunqian Li

Abstract Stem cells provide a promising therapy for treating stroke. Several imaging techniques provide the possibility of the visual representation of biological processes of transplanted stem cells in vivo. Further means of monitoring the transplanted cells safely, noninvasively, and longitudinally would contribute to the understanding of the underlying stem cell therapeutic mechanism. In this chapter, we describe the state-of-the-art methods of monitoring transplanted stem cells in vivo, including magnetic resonance imaging (MRI), radionuclide imaging, and optical imaging. Their principles vary and each has advantages and drawbacks. Presently, no single technique is excellent through ideal criteria; the combination of multiple imaging modalities is thus an attractive strategy.

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Keywords Stem cells • Stroke • Imaging • Magnetic resonance imaging • Radionuclide imaging • Optical imaging

14.1 Introduction

In spite of considerable advances in stroke management, current acute treatments have only mild effects and moderately restore its lost function [1, 2]. Therefore, stroke remains a major cause of disability and needs much more efficient therapeutic approaches. Cell transplantation is a novel treatment method in many fields of medicine, such as the cell replacement in the ischemic region to prevent further stroke disability [3]. In the last decade, evidence of neurogenesis in the adult human brain has provided the basic scientific hypothesis of cell transplantation therapy for various neurological disorders, including Parkinson's disease, multiple sclerosis, and stroke. Initial animal and experimental studies have identified a significant benefit of stem cell transplantation in neuroregeneration and improvement in neural function [4, 5]. Stroke is one of the most severe neurological disorder, which has been selected for a pioneering trial in the clinical application of stem cells [6, 7]. Furthermore, several studies demonstrated the feasibility of stem cell-based therapy for the restoration of lost brain function and improvement of the clinical outcome in stroke patients [2]. Several experimental and clinical researches have introduced different types of stem cells for transplantation in stroke [8, 9]. Moreover, clarification of optimal cell dosing, route of transplantation, cell delivery methods, and in vivo cell imaging techniques is needed to ensure safety, efficacy, expected outcome, and more success of potential stem cell transplantation trials [10].

Nowadays, radionuclide, fluorescence, and MRI are suitable candidates for human nervous system cellular imaging [11–13]. Among them, positron emission tomography (PET) is more sensitive to low concentrations of contrast agents. However, it has some limitations such as low spatial resolution, radiation exposure, and short-term signal production. Another technique is fluorescence, which is a sensitive method with some distinct advantages in small animal models, but it is not feasible for human whole-body visualization because of the limited penetration depth and low spatial resolution [12]. Although a high spatial resolution can be provided by other methods like micro-CT, this technique is not always suitable for in vivo human studies, and it needs to be optimized for better cell detection throughout the whole body. With respect to the full commitment of clinical studies and trials to patients' safety, radiation and radioactive exposures are important limitations of CT, PET, and single-photon emission computed tomography (SPECT). Therefore, MRI is a superior method for cell tracking and imaging. Several stem cell tracking studies have been performed using MRI, and the most popular area is neurological diseases (Table 14.1) [14].

Table 14.1 Comparison of several in vivo cell imaging modalities

Modality	MRI	Radionuclide	Fluorescence
Radiation injury	No	Yes	No
Acquisition time	Microseconds to hours	Minutes	Minutes
Depth	No limit	No limit	<1 cm
Resolution	10–100 μm	1–2 mm	2–3 mm
Longitudinal cell tracking	+++	+	+++
Sensitivity	+++	+++	+
Clinical application	Yes	Yes	No
Quantification of cell number	Possible	Yes	No
Assessment of cell viability	Possible	Yes	Yes

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14.2 Magnetic Resonance Imaging (MRI)

Hongbin Han, Wei Wang and Chunyan Shi

MRI is a promising noninvasive in vivo stem cell tracking technique, and its primary advantage is the safety and clinical applicability without radiation and radioactive exposures. Furthermore, the living environment of brain cells can be quantitatively measured using a novel tracer-based MRI [15, 16]. Therefore, it is of tremendous value to dynamically monitor transplantation, migration, and therapeutic response over a long period of time. Based on the radio wave, MRI has no limit to tissue depth penetration. MRI can display three-dimensional information of the brains; meanwhile, arbitrary section images can be obtained. MRI can provide detailed information on host organs with superior soft tissue contrast and excellent spatial resolution that can reach below 50 μm and even single-cell detection under ideal conditions [17]. MRI also can provide multiparameter information, which are associated with relaxation time, proton density, flow, or biochemistry.

14.2.1 Concepts and Principles

MRI has been used as a medical diagnostic tool for more than 30 years, and the first image was published in 1973 by Lauterbur who received the 2003 Nobel Prize in Physiology or Medicine [18]. The principle of MRI is based on radio frequency emitted when selective atomic nuclei are placed in an external magnetic field, in which hydrogen atoms are most commonly used. The radio-frequency signal can be detected by the extracorporeal coils. A variety of parameters of the body's tissue can then be calculated and images are generated. Presently, two strategies are reported for tracking stem cell using MRI. Firstly, stem cells can be tracked using exogenous metal-based compounds and endogenous genetic-based agents, both of which can modify the relaxation time of radiowaves of the surrounding hydrogen protons.

Secondly, stem cells can also be labeled with selective atoms such as fluorine-19, which emits a characteristic radio frequency. The modified radio-frequency signals can be detected by MR and generate contrast in images; therefore, stem cells can be distinguished from the background once implanted in the brain.

14.2.2 Methods and Tracers

Metallic tracers are primarily developed among cell tracking strategies. The first studies of MR cell tracking in the brain were reported in 1992, in which transplanted cells were labeled and tracked using superparamagnetic iron oxide (SPIO) [19]. Over the years, a variety of metal-based tracers have been developed and have more efficiency in labeling and tracking the implanted cells. According to the effect on relaxation time or signal density, these metal-based tracers can be classified into positive and negative groups. Positive tracers can decrease the longitudinal/T_1 relaxation time and increase the MRI signal density on the T_1-weighted image. Presently, positive tracers mainly involve gadolinium (Gd^{3+}) and manganese (Mn^{3+}) chelates, while negative tracers can decrease the transverse/T_2 relaxation time and the MRI signal density on the T_1- or T_2^*-weighted image. Negative tracers are primarily superparamagnetic iron nanoparticles (SPIOs).

14.2.3 Application and Limitation

14.2.3.1 Positive Tracers

Positive tracers are paramagnetic agents, which have one or more unpaired electrons. Gd^{3+} chelates are the most effective paramagnetic agents, owing to their seven unpaired electrons. Gadolinium-diethylenetriaminepentaacetic acid (Gd-DTPA) is the most common contrast agent in clinical application. Tracers for stem cell labeling include Gd-chelated dextran nanoparticles and Gd-DOTA-peptide complexes and Gadolinium (Gd^{3+})-doped mesoporous silica nanoparticles [20]. However, the problem using gadolinium to track stem cells is the risk of toxicity on cells and tissues. Free ionic gadolinium is highly toxic; Gd-DTPA, an FDA-approved agent, can elicit nephrogenic systemic fibrosis [21]. Presently, because of the low sensitivity of Gd-based agents and low uptake of Gd^{3+} chelates by targeted cells, enough Gd^{3+} chelates are necessary for labeling cells to generate efficient contrast on images, which may increase the risk of toxicity to cells and host tissue [18]. For example, in a rat stroke model, transplants of stem cells labeled with gadolinium-rhodamine dextran resulted in a significant increase in lesion size compared to the groups with fluorescent dye labeling [22]. The exact mechanism of gadolinium toxicity is not known, but a possibility is that free Gd^{3+} can influence cell metabolism of the labeled cells through distorting the activity of K^+/Cl^- membrane cotransporter [23]. It is also

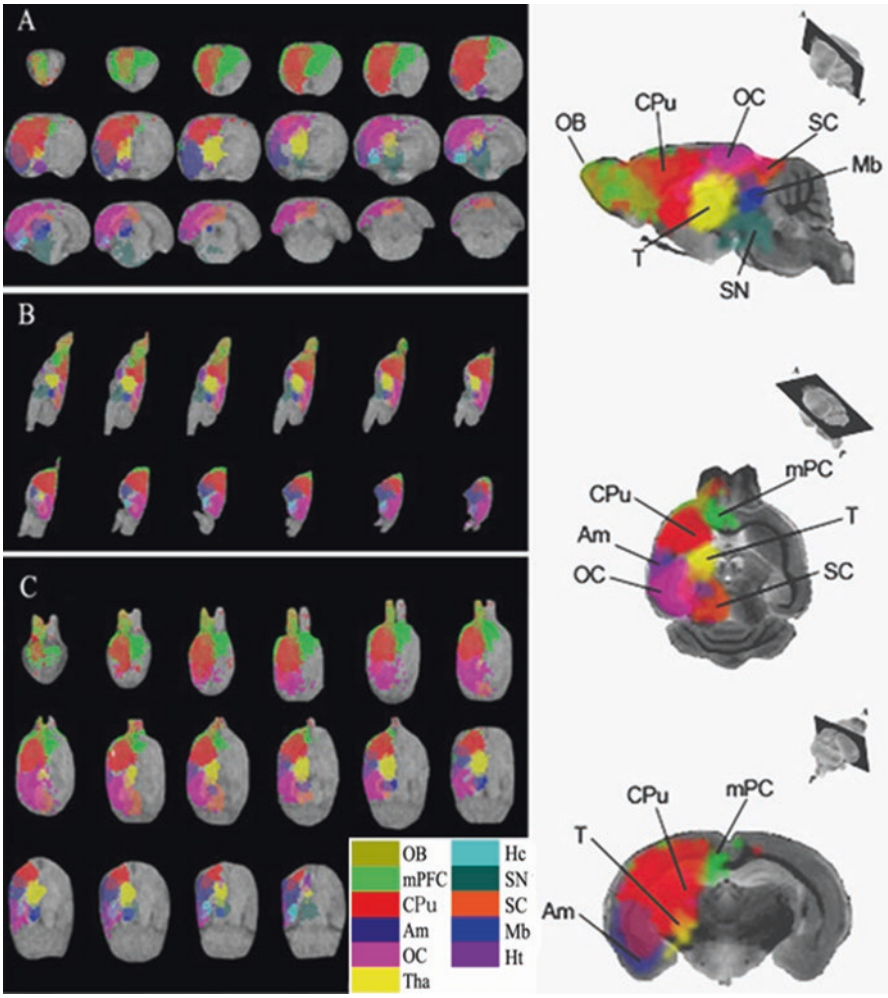

Fig. 14.1 Color map of the brain extracellular space divisions based on the brain interstitial space flow. Each colorized area represents the $Volume_{max}$ of the traced interstitial space flow with 11 different start points: coronal (**a**), sagittal (**b**), and transverse views (**c**) (area 1, *OB* olfactory bulb; area 2, *mPFC*, medial prefrontal cortex; area 3, *Cn*, caudate putamen; area 4, *Am*, amygdaloid nucleus; area 5, *Oc*, occipital cortex; area 6, *Tha*, thalamus; area 7, *Hc*, hippocampus; area 8, *SN*, substantia nigra; area 9, *SC*, superior colliculus; area 10, *Mb*, midbrain; area 11, *Ht*, hypothalamus)

unclear how these Gd^{3+} are metabolized in labeled cells. Recently, chelated gadolinium was employed to trace the interstitial or extracellular space in the deep brain [15] where in it provides the living environment of both the host and the implanted brain cells (Fig. 14.1). The new discovery of transportation barrier in the brain interstitial space has great implications for the site of implantation [16, 24].

Manganese is another paramagnetic tracer for positive T_1 contrast of labeled cells, owing to their five unpaired electrons. It was used as the first MR contrast agent in 1978. The behavior and metabolism of Mn^{2+} ions in the cell is similar with those of calcium (Ca^{2+}) ions and both enter cells through ligand- or voltage-gated Ca^{2+} ion channels. Therefore, Mn^{2+}-enhanced MRI has been performed to investigate neuronal activity and to visual neuronal connectivity. However, regarding the potential cytotoxicity, a few reports exist concerning the use of manganese-based agents to stem cell tracking. Silica-coated MnO nanoparticles have been developed for the labeling and MR tracking of mesenchymal stem cells [18].

14.2.3.2 Negative Tracers

Negative tracers are superparamagnetic agents. They can cause magnetic resonance signal dephasing related to local magnetic field inhomogeneity induced in water molecules near superparamagnetic particles (susceptibility effect) and shorten transverse relaxation times of hydrogen protons. As a result, labeled stem cells produce dark spots in T_1- or T_2^*-weighted images. Superparamagnetic iron oxide (SPIO) particles with various diameters are the most common negative tracers. SPIO is typically composed of a Fe_3O_4 or Fe_2O_3 core with varying chemical coatings, such as dextran, glycosaminoglycans, starch, polyethylene glycol, siloxane, and polylactic acid. They can be categorized according to size. Ultrasmall superparamagnetic iron oxide particles (USPIO) measure less than 50 nm in diameter. SPIO particle size varies from 50 to 200 nm, and micronized paramagnetic iron oxide (MPIO) particles measure more than 1 μm in diameter. SPIOs were first reported for the purpose of labeling and tracking transplanted rat brain cells in 1992–1993 and demonstrating cell migration in vivo in 2001. Among them, ferumoxide, an iron oxide nanoparticle coated with dextran, was approved by the FDA in 1995 as a MRI enhancement contrast agent for the clinical diagnosis of liver lesions. Since then, ferumoxides have been widely applied in cell labeling, although it is no longer being made due to the lack of sales. Presently, no SPIO has gained FDA approval for labeling stem cells. Other metal-based negative tracers such as bimetallic ferrite nanoparticles (e.g., $CoFe_2O_4$, $MnFe_2O_4$, and $NiFe_2O_4$), hybrid magnetic nanoparticles, and iron-platinum-based nanoparticles have the potential for high biocompatibility, sensitivity, and detectability, but are still in the early trial stages and only utilized in the lab.

Advantages of SPIO over conventional paramagnetic tracers are as follows:

Firstly, SPIO has a much stronger MR relaxivity and higher sensitivity. Gadolinium chelates can only be detected in the micromolar range, while SPIO is sensitive in the nanomolar range for visualizing single-cell tracking and is suitable for molecular imaging [25].

Secondly, the concentration level of SPIO for labeling stem cells has negligible cytotoxicity. Transfection agents have been used in conjunction with SPIO for labeling stem cells, which may be toxic at certain levels either through cell death

or prevention of cell division. Currently, some alternative coatings have been developed to facilitate the administration of SPIO to cells without the use of a transfection agent, which significantly decreases the local toxicity to cells due to iron crossing both nuclear and mitochondrial membranes [26].

Thirdly, iron-containing SPIO shows an acceptable level of biocompatibility in part due to the body's innate ability to metabolize naturally occurring iron in the form of ferritin [27].

While SPIO is the most common tracer for magnetic cell labeling, there are some limitations:

Firstly, SPIO can produce the blooming effect, that is, "dark spots" in images are beyond the volume occupied by SPIO. The effect is helpful in increasing the visibility of the labeled stem cells however, it interferes with visualizing host anatomical structures and provides false locations of the labeled cells [28].

Secondly, with stem cell division and proliferation, the ratio of cells labeled with SPIO decreases. As a result, longitudinal tracking will result in gradual signal loss [29].

Thirdly, iron released from apoptotic or lytic cells can be internalized by macrophages in nearby tissues, resulting in signal not associated with the target cells [30].

Fourthly, besides SPIO, many factors, including bleeds, air, and hemorrhage, can present false-positive hypointensities on T_1 and T_2^* images [17].

Fifthly, SPIO has limited capability in reporting cell functionality and viability and is insufficient to attain comprehensive information of stem cell post transplantation. Histology has shown that hypointensity persists even after labeled cell death. This highlights the necessity of some external modality to confirm that the labeled cells are viable and unimpaired [28].

Sixthly, quantifying labeled cells is challenging. In the relaxometric-based experiments, relaxivity changes depend on physical properties of internalized SPIO during degradation. Signal intensity is not directly proportional to the concentration of SPIO and cannot be related back to cell numbers.

Lastly, SPIOs are generally considered as non-toxic. However, due to the complexity and variety of components of SPIOs, their toxicity profiles are still controversial. Ramaswamy reported successful labeling of mesenchymal stem cells (MSCs) using Feridex® without impairing cell chondrogenesis differentiation, while Bulte reported that chondrogenic differentiation of MSCs is inhibited after labeling with Feridex® [29].

14.2.4 Conclusion and Future Perspectives

Safety is paramount in the implementation of tracers for tracking stem cells. The ideal magnetic tracer must be nontoxic to cells, biodegradable, effective in low doses, chemically stable in the body, not affect stem cell function, must not transfer harmful by-products nor affect stem cell function [27]. Therefore, it is necessary to

fully characterize tracer effects on stem cells (i.e., cytotoxicity), including viability, differentiation, migration/homing, distribution, and engraftment [29]. Cytotoxicity is related to the potential for organismal migration, penetration, and accumulation of metal. There are several developing techniques for eliminating cytotoxicity. Firstly, the use of a tracer based on ^{19}F rather than metal-based tracers, allows for quantitative cell tracking. In ^{19}F MR images, the background signal is lacking and an accurate, unambiguous detection of labeled cells can be achieved. The relationship between the concentration of ^{19}F and signal intensity is directly proportional and linear, and the labeled cell numbers can be quantified directly from the acquired images. Unfortunately, ^{19}F-based tracking MRI is poorly sensitive, and the minimum amount of cells that can be detected ranges from 10^3 to 10^5 cells [18]. Secondly, developing a high relaxivity tracer is necessary to achieve high cellular magnetization, so that less metal is required to obtain the same contrast. Indeed, given sufficient magnetization, in vivo single-cell detection is possible, while tracers in very low doses may have negligible side effects [31].

An ideal magnetic tracer should allow for longitudinal tracking of implanted stem cells for follow-up of its viability and function. Longitudinal tracking using a tracer is necessary for studying the efficiency of stem cell therapy. Conventional magnetic tracers are unsuitable for longitudinal tracking due to signal loss resulting from stem cell division and proliferation. Recently, novel methods using reporter genes are reported for use in longitudinal tracking. Reporter genes are DNA sequences that are introduced to the stem cell genome. The reporter genes encode reporter proteins like ferritin and tyrosinase. These reporter proteins accumulate intracellular iron, which allows for a paramagnetic effect to be detected on MRI. As the reporter genes are stably expressed in the host cell genome, the tracer will not be lost or diluted even after cell division. Furthermore, the signal emitted from reporter genes is based on the viability of the host cells. Thus, reporter genes can be used simultaneously for longitudinal tracking and viability detection [18, 20, 32–34].

The ideal magnetic tracer-based imaging for stroke should also be characterized as follows: Use of imaging in clinical trials is strongly encouraged to provide as much information as possible to assess vascular/structural lesions, infarct size, cell viability, location, the success and safety of implantation, and inflammation. Imaging should also be used to monitor safety and recovery and, when possible, investigate mechanisms of action and provide information on surrogate markers of treatment effect. Imaging measures might also be useful to help stratify patients at baseline [35]. Because no single technique fufills all these criteria, the combination of multiple imaging modalities is an attractive strategy. Multimodal imaging can exploit the strengths and overcome limitations and has been developed rapidly over the last decade [26]. Gadolinium and SPIO may be combined with the fluorescent compound allowing their detection by histology in vitro and by MRI in vivo [36, 37]. PET for high sensitivity and specificity coupled with MRI for high spatial and temporal resolution has been applied for cell tracking following ischemic stroke [17]. Magnetic nanoparticles can be conjugated to identify molecular sensors that detect stimuli associated with cell viability and functions, such as chemicals secreted during cell differentiation, physical contact with neighboring cells during stem cell

engraftment, intercellular pH changes during cell death, and certain molecules related with stem cell differentiation. The interaction between the sensors and the stimuli generates detectable changes of signal in the magnetic nanoparticle that can be captured by MRI [29, 38].

14.3 Radionuclide Imaging

Weifang Zhang

Among the different imaging techniques available, nuclear medicine has become one of the most employed techniques, due to its favorable characteristics, such as the availability of different radiopharmaceuticals, high sensitivity, good tissue penetration, and translation to clinical applications [39]. Because of their exquisite picomolar (10^{-11} to 10^{-12} mol/l) sensitivity, radionuclide imaging modalities are able to detect trace quantities of radioisotopes for studying biological processes in living subjects. Radiopharmaceutical cell labeling has been used for decades to systemically monitor cells in nuclear medicine studies, such as labeled leukocyte scintigraphy for the detection of infectious and inflammatory diseases. Besides its high sensitivity, radionuclide imaging has several advantages. It is highly quantitative, which means it is dynamic and kinetic, observing the biodistribution of implanted cells in vivo. In fact, radionuclide imaging is the sole direct labeling technique used thus far in human studies, involving both autologous bone marrow-derived stem cells and peripheral hematopoietic stem cells. Technological developments of both PET and SPECT have further facilitated the implementation of specialized systems for small animal imaging with much greater spatial resolution (1–2 mm) to emerge as one of the most powerful tools and dramatically advance the field of in vivo cell tracking.

14.3.1 Concepts and Principles

A variety of labeling methods with radionuclides have been created and used to study cell distribution in the body. Technetium-99m (^{99m}Tc) is currently the most used radionuclide in the world due to favorable properties, such as its decay by gamma emission with an energy of 140 keV and a 6 h half-life, optimum physical characteristics for SPECT, allowing images for up to 24 h after injection. ^{99m}Tc has wider availability and lower cost compared with other radionuclides, with good resolution and low radiation dose to the patient and to the labeled cells [40]. Radionuclide indium-111 (^{111}In) may also be used for cell labeling in SPECT, which allows cell tracking for up to 96 h but results in lower-resolution images and a higher radiation dose to the patient and labeled cells. In addition, different studies have indicated that Auger electrons of ^{111}In-oxine labeling affect cellular integrity and lead to cytotoxicity of stem cells.

The radionuclide fluorine-18 (^{18}F) has a half-life of approximately 110 min and is the most commonly used in positron emission tomography (PET) and hybrid PET/CT, mainly in the radiopharmaceutical ^{18}F-fluorodeoxyglucose (^{18}F-FDG). ^{18}F-FDG allows cell labeling and tracking for a few hours. ^{18}F can also be incorporated into a modified thymidine analog, 3′-deoxy-3′-^{18}F-fluorothymidine (^{18}F-FLT), which is phosphorylated by thymidine kinase but not incorporated into DNA, thus sequestering ^{18}F-FLT within the cell. ^{18}F-FLT has been used to visualize neural stem cell mobilization in the mouse hippocampus. Unlike SPECT scans, PET has a two- to threefold higher spatial resolution than SPECT (3–6 mm versus 10–15 mm) and allows quantification of standardized uptake values, which may be used to compare the response to different therapies. Zirconium-89 and Cu-64 are other promising radionuclides for cell labeling in PET that possess longer half-lives (78.4 h and 12.7 h) and may allow cell tracking for several days to weeks.

Tracking cells with SPECT and PET may be separated to two strategies: direct and indirect. Direct tracking is that stem cells are labelled with a radiotracer in vitro prior to transplantation in vivo, while indirect. Indirect cell tracking may be carried out via reporter gene/probe systems.

14.3.2 Methods and Tracers

In general, radioisotopes with a relatively long decay half-life are used to track cells for a period of several hours or even days. These can be done with radiopharmaceuticals such as ^{99m}Tc-hexamethylpropyleneamine oxime (^{99m}Tc-HMPAO) or ^{111}In-oxine for SPECT and ^{18}F-FDG for PET. The isotope is carried into the cells via a lipophilic chelator, which governs the initial extraction of the tracer into the cells. Once inside the cells, a trapping mechanism reduces the lipophilicity of the molecule, and the isotope is retained. After a short incubation period, the cells are washed to remove any unbound activity and are injected into the host.

Both SPECT and PET imagings offer visualization of radiolabeled cells or tissue structures with high spatial resolution. However, because of the short half-lives of most radioisotopes used, one cannot track the long-term survival and fate of these cells. Another major concern is that direct radionuclide labeling cannot accurately distinguish live from apoptotic cells because radioisotopes will remain active even after cell death or may leak from dead cells into surrounding non target cells.

The concept of reporter gene for PET/SPECT detection involves the construction of a deoxyribonucleic acid sequence coding a specific reporter. A vector delivery system is used to carry the reporter gene into cells of interest. The expression of the reporter gene product in the cells, when exposed to the corresponding radiolabeled reporter probes, combined specifically with the probes, “turns on” the imaging signal in the probe and entraps the activated probe inside the cells. Finally, imaging and recording of the signal are achieved by use of appropriate devices, such as PET, SPECT, or multimodal device [41, 42]. Because accumulation of the reporter probe requires expression of the reporter gene and activity of the reporter-gene product, the imaging signal

will be dependent on viability of the therapeutic cells. This approach could track transplanted cells in the human body as long as they remain alive and would also be suitable for assessing the proliferative capacity of the transplanted cells. This is in contrast with direct labeling techniques and provides a more specific readout. Additionally, imaging can be performed repeatedly and is not limited by radioactive decay of the initial label load.

Reporter gene/probe systems have traditionally been divided in three groups, according to the way that the protein product of the reporter gene interacts with the reporter probe and causes its accumulation on the surface or inside the cells: (1) reporter genes that encode enzymes to phosphorylate specific reporter probes leading to their entrapment; (2) reporter genes that encode protein receptors, which in turn bind to specific reporter probes; and (3) reporter genes that encode cell membrane transporters that accelerate the accumulation of reporter probes in the cells. The best known and most widely used reporter gene for both PET and SPECT imaging is the herpes simplex virus type 1 thymidine kinase gene (*HSV1-tk*). A lentivirus may be used to deliver a reporter gene for the expression of herpes simplex virus-truncated TK that catalyzes a reaction leading to the accumulation of the probe. Herpes thymidine kinase can phosphorylate two main classes of exogenously administered reporter probes: pyrimidine analog derivatives, such as 2′-fluoro-2′-deoxy-β-D-arabinofuranosyl-5-iodouracil (FIAU), and acycloguanosine derivatives, such as 9-(4-fluoro-3-hydroxymethyl-butyl)guanine (FHBG). When herpes thymidine kinase phosphorylates its reporter probe, not only is the probe retained within viable cells but signal amplification also occurs when the enzyme reacts with multiple substrates. To facilitate either PET or SPECT imaging, reporter probes can be synthesized with the appropriate isotope e.g., positron-emitting fluorine-18 and iodine-124 or gamma-emitting iodine-123, iodine-125, and iodine-131 [43].

Although reporter gene imaging has become very widespread in preclinical studies, few studies have shown a proof-of-principle application for cell therapy in the human [44] (Fig. 14.2). Reporter gene imaging requires genetic modification of transplanted cells, which increases the regulatory complexity for their approval and poses additional risks for mutagenesis. Furthermore, the radioactive tracers would expose the patient to higher radiation doses. However, in the case of MSCs, it has been shown that free radical scavenging and DNA repair mechanisms are particularly robust, allowing for higher radiotolerance.

14.3.3 Application and Limitation

Among the various methods available, radioisotope cell labeling has become one of the most promising since it permits tracking of cells after injection by different routes to investigate their biodistribution for neurological diseases.

Studies using radiopharmaceuticals to track the fate of transplanted cells have given important clues about the mechanisms of action of cell-based therapies for neurological diseases. In many of these studies, the transplanted cells exerted therapeutic

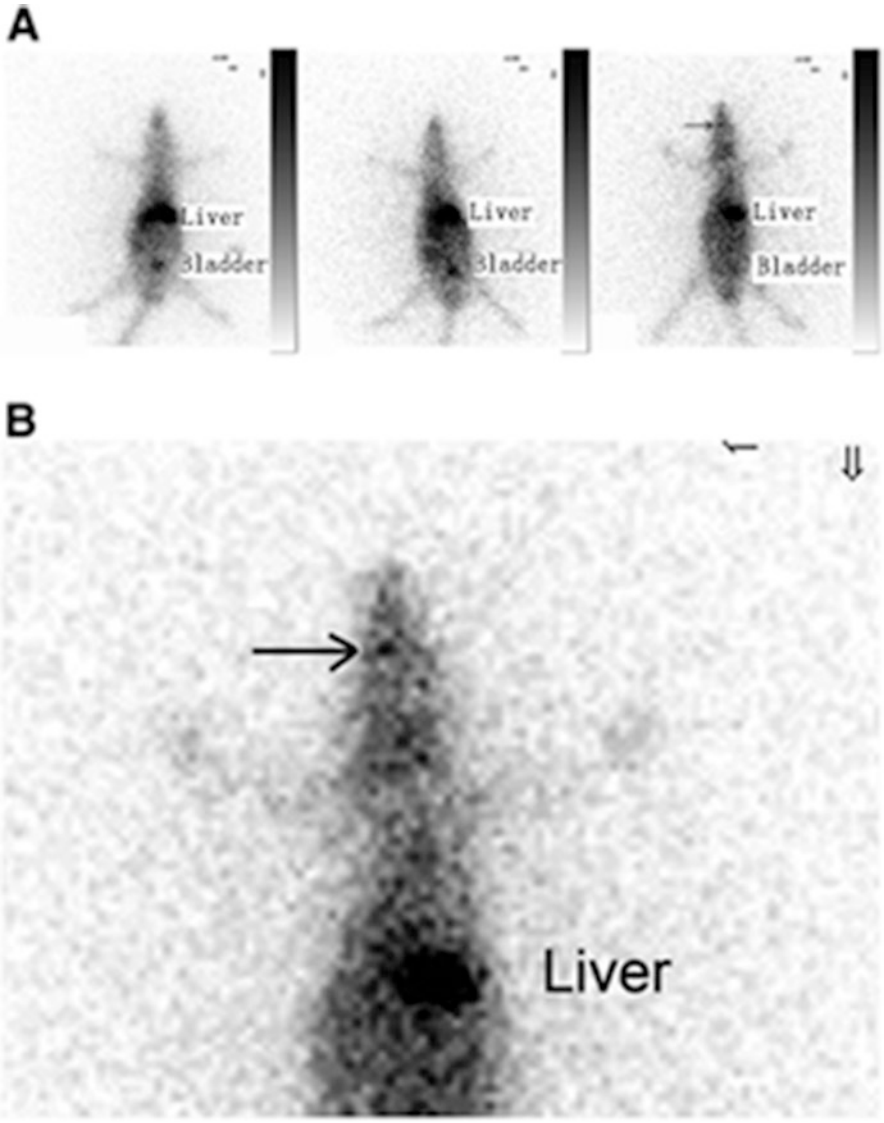

Fig. 14.2 SPECT HSV1-tk-^{131}I-FIAU reporter gene images of experimental middle cerebral artery occlusion rat models with transplanted *BMSCs*. **a** Liver and bladder were clearly imaged. Level of accumulation in brain (*arrow*) was low but increased gradually over time. **b** Local enlargement of A. Greater radioactivity accumulation was seen at cell injection site (*arrow*) (Reprinted and adapted with permission from J Nucl Med. Ref. [44])

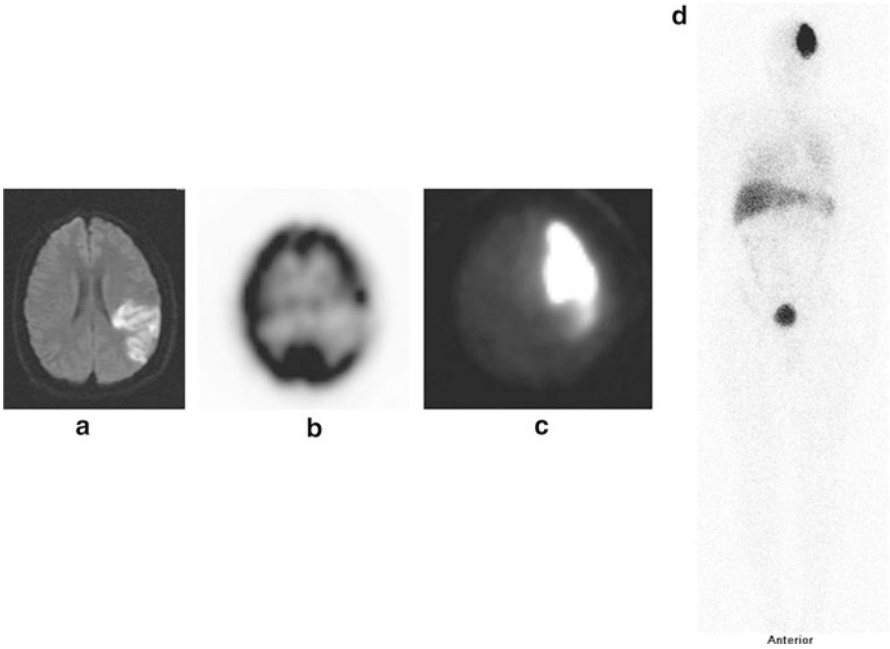

Fig. 14.3 The accumulation of radiopharmaceutically labeled stem cells in stroke. **a** Diffusion MRI showing acute ischemic lesion in the left middle cerebral artery territory. **b** Brain perfusion Tc-99m ECD SPECT showing left temporoparietal hypoperfusion. **c** Tc-99m HMPAO autologous mononuclear bone marrow cell (ABMMN) brain SPECT revealing accumulation of the ABMMN-labeled cells in the left brain hemisphere, more intense in the anterior region. **d** Anterior whole-body scan with Tc-99m HMPAO ABMMN cells revealed left brain, liver, and spleen uptake. There was no lung uptake (Reprinted and adapted with permission from Clin Nucl Med. Ref. [45])

effects by limiting tissue damage and/or by stimulating regeneration and plasticity of the diseased central nervous system. In recent years, bone marrow mononuclear cells (BM-MNCs) remain the primary source of stem cell therapy for treating neurological diseases. ^{111}In-oxine, ^{99m}Tc or ^{99m}Tc-HMPAO, ^{131}I-FIAU, ^{18}F-FHBG, and ^{18}F-FDG are mainly used in several preclinical and clinical trials to track transplantated cells for different neurological diseases, including spinal cord and brain injury, transient cerebral artery occlusion, cerebral ischemia, and ischemic stroke. Research shows that transplantation of radiopharmaceutically labeled stem cells led to accumulation in the site of injection in animal models of spinal cord or brain injury. In contrast, intravenous and intra-arterial injection led to little homing to the brain or spinal cord, and biodistribution was mainly to the liver, spleen, lungs, and kidneys [45] (Fig. 14.3). Although the number of transplanted cells that accumulated at the lesion site was very low in most cases, recent studies have clearly shown that BMSCs may promote functional recovery after various kinds of central nervous system disorders, including ischemic stroke. Using an ^{18}F-FDG, PET/CT apparatus clearly demonstrates that the BMSCs not only enhance functional recovery but also promote the recovery of glucose utilization in the peri-infarct area after ischemic stroke [46] (Fig. 14.4).

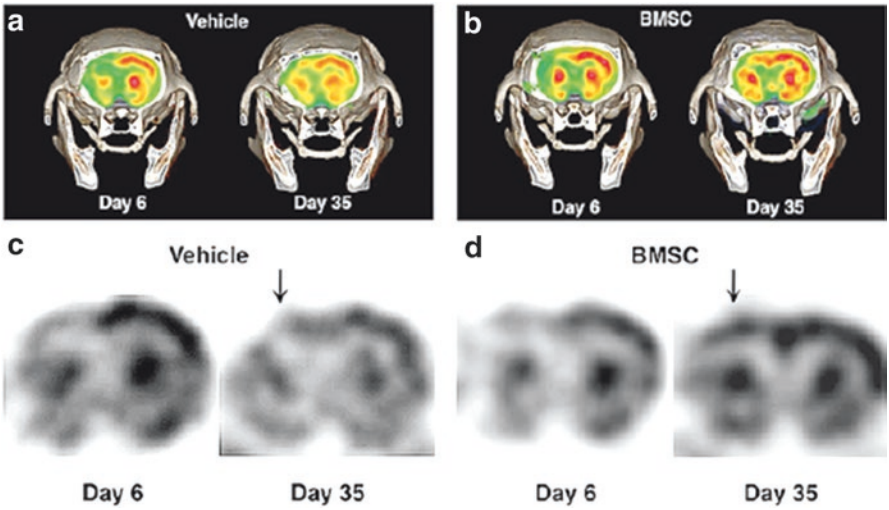

Fig. 14.4 Representative findings of 18F-FDG PET at 6 and 35 days after ischemia. Color (**a**) and *black*-and-*white* (**c**) images of vehicle transplanted animals. Color (**b**) and *black*-and-*white* (**d**) images of *BMSC*-transplanted rats. There was significant increase in local glucose metabolism in the peri-infarct neocortex (*arrows*) (Reprinted and adapted with permission from J Nucl Med. Ref. [46])

Nuclear techniques, including reporter genes and direct cellular radio labeling, afford very good detectability but more limited spatial resolution. Rapid advances in imaging modalities have now resulted in the development of fused imaging systems, such as PET/CT, SPECT/CT, PET/MRI, and even dual-mode systems e.g., MRI/SPECT (PET), improving both sensitivity and specificity significantly. Multimodal imaging is expected to achieve more accurate information of the transplanted cells. In addition to a more precise localization of the homing site, the fusion of nuclear medicine images with CT or MRI allows evaluation of different aspects, such as (1) correlation of cell homing with positive morphological and functional effects, (2) evaluation of adverse reactions including brain hemorrhage or formation of tumors, and (3) the effect of different doses and routes of injection on cell migration and proliferation [47].

Cicchetti et al. combined labeling subventricular zone- (SVZ-) derived neural stem/progenitor cells with SPIOs to evaluate their migration with MRI and simultaneously utilized different radiotracers to analyze physiological aspects with PET [48]. Tang et al. synthesized an MRI/SPECT/fluorescent trimodal probe (125I-fSiO4@SPIOs) for quantitatively tracking MSCs transplanted into stroke rats. They labeled the probe with SPIOs and radioisotope simultaneously, which allowed them to track the labeled MSCs in vivo with high spatial resolution and anatomical localization by MRI and high sensitivity by SPECT. The study demonstrates that 125I-fSiO4@SPIOs are robust probes for long-term tracking of MSCs in the treatment of ischemic stroke [49].

14.3.4 Conclusion and Future Perspectives

Methods for cell tracking with radioisotopes are feasible and efficient, and different studies have used it to monitor migration in cell therapies for neurological diseases. These techniques provide validated quantifications of cell retention in different organs and the dynamics of cell distribution in the whole body. The potential value of the information that radionuclide imaging could provide may justify the use of this technique in the future. The combination of different imaging modalities allows for cell tracking in conjunction with assessing cell metabolism non-invasively, and such methods have the potential of answering important questions in the field of stem cell therapy. PET/MRI will likely emerge as the imaging modality of choice given that it provides exquisite functional and anatomical detail with minimal radiation exposure.

14.4 Optical Imaging

Lan Yuan, Junhao Yan and Yunqian Li

Optical imaging is an easy, fast, and inexpensive tool for monitoring cell transplantation therapy in vivo. Optical imaging encompasses a variety of cell imaging modalities including fluorescence imaging and bioluminescence imaging (BLI). All of these methods involve the detection of photons emitted either by chemical oxidative processes or by external excitation of a fluorophore [17]. The technique is usually based on ex vivo labeling of the donor cells with a fluorescent dye [50]. Compared with other cell imaging technologies, the advantages of fluorescent optical imaging originate from its non-invasiveness, real-time in vivo imaging and high repeatability, sensibility, and security.

14.4.1 Concepts and Principles

Optical imaging of stem cell tracking is a cell imaging technology that introduces an exogenous fluorescence probe; a fluorescence microscope can then be used to trace the implantation, migration, and survival of stem cells and evaluate the clinical effects. Aptamers are single-stranded RNA or DNA oligonucleotides usually 15–60 bases in length that bind specifically to target molecules. Typically, aptamers can be generated from a selection process termed as systematic evolution of ligands by exponential enrichment (SELEX) [51, 52]. Aptamer labeling can alternatively be applied to monitor the differentiation process of stem cells, as shown by Iwagawa et al. [53]. Their selected aptamers showed specific binding affinity to mouse embryonic stem cells (mESCs), with low affinity toward differentiated cell lines. During the course of retinoic acid (RA) differentiation, multiple injections of the aptamer

showed declining cell affinity with a progressive decrease in the fluorescent signal. Applying the same principle, stem cells can potentially be incubated and tagged with similar aptamer probes prior to their injection to facilitate colorimetric evaluation of successful cell differentiation [54]. Through external excitation, the fluorescent light group emits the fluorescence after reaching its high energy state [55]. The intensity of fluorescence can reflect the signal strength of stem cells, which is determined by the transplanted stem cells in the body of their implantation, migration, and survival situations. The stem cells labeled with fluorescent dyes were transplanted into the mice and then real-time tracking of the transplanted stem cells was conducted using the fluorescence microscope. BLI is the most well studied of the various optical imaging modalities with regard to stem cell imaging in the brain. Bioluminescence involves transducing a reporter gene, which codes for firefly luciferase or Renilla luciferase, into the stem cells [17]. When the luciferase enzyme reacts with its substrate D-luciferin or coelenterazine, it emits photons, which can then be detected and quantified by a charge-coupled device camera system [56].

14.4.2 Methods and Tracers

Nowadays, much more attention has been paid to the application of using in vivo optical imaging to track the transplanted stem cells in real-time. In fact, there are various fluorescence probes of labeling and tracking stem cells.

Green fluorescent protein (GFP) was first found in *Aequorea victoria* by Shimomura in 1962 and has been widely used to label various kinds of cells through transgenic or transfection [57]. The discovery paved the way for a universal marker for cell structures and cellular processes detectable by fluorescence microscopy. The diversity of FPs has increased tremendously by mutating the original GFP sequence and cloning FPs from distant species like crustaceans. Such FPs can be expressed in mammalian cells, including stem cells and transgenic mice without signs of toxicity. Furthermore, smart multi-label approaches like the Brainbow toolbox have been developed to mark neurons with many different FPs [51]. Under the blue wavelengths of light, GFP can emit green fluorescence. At present, the tracer-labeled cell imaging technology has been extensively used in some animal models. Yang et al. had injected mouse melanoma cells that expressed a high level of GFP into the tail vein or portal vein; the results indicated that the metastatic lesion in the brain, liver, and bone could be clearly visualized using whole-body optical images [58]. In addition, Hideo Shichinohe et al. also transplanted stem cells labeled with GFP into mouse brains to trace the conditions of stem cells [57].

The quantum dot (QD) is a type of near-infrared fluorescent dye with good light stability. Additionally, its fluorescent intensity is 20 times higher than that of traditional organic fluorescence dyes e.g., rhodamine. QDs consist of an inorganic core, a shell of metal, and an outer organic coating with a total diameter of 2–10 nm and are quickly becoming very important for in vivo imaging [17]. Therefore, researchers can observe QD-labeled cells for a long time, and it has been a favorable tool for

studying the interaction between biological macromolecules in the cell. Due to its longer wavelengths, the near-infrared (NIR)-emitting QDs can easily penetrate the tissues, including the bone and skin. QDs can be synthesized to the desired specifications, including size, shape, and photon emission energy [17]. They are currently regarded as useful biological probes e.g., labeling the bone mesenchymal stem cells (BMSCs) owing to their nanometer dimensions, attractive optical characteristics, high resistance to bleaching or degradation, and strong fluorescence. QD-labeled BMSCs have been transplanted into the infarcted rat brain and the fluorescence can be tracked for at least 8 weeks after transplantation [59]. Unfortunately, cell labeling with QDs suffer from the same long-term in vivo imaging difficulties as direct labeling in that the signal will be diluted due to cell proliferation. Near-infrared-emitting QDs have a potential for in vivo cell tracking within the brain, as demonstrated by Kawabori et al. [60].

14.4.3 Application and Limitations

Optical imaging has some advantages, such as the lower cost, non-radiative, rapid acquisition, high sensitivity, etc. Therefore, it has been widely applied for in vivo imaging in biological tissues [59]. In recent years, with the in-depth study of stem cells, fluorescence imaging techniques has been extensively used to trace the survival and migration of transplanted stem cells. These aptasensors rely on the highly specific, structure-switching ability of aptamers; they undergo drastic secondary or tertiary folding from their initial conformation upon binding with their target molecules [61]. By labeling aptamers with quencher and fluorophore dyes at their 5′ and 3′ ends, a target-binding event, which causes a displacement of the two dyes, can be transduced to a change in fluorescent signal as a result of Förster resonance energy transfer (FRET) principles [54]. Serial optical fluorescence images in vivo could identify the distribution of cells in the cerebral cortex and demonstrate real-time cell migration in the animal. The optical fluorescence imaging in vivo can provide important information regarding the behaviors of donor cells through non-invasive and serial visualization [50].

Apart from the optical imaging technology, other imaging techniques such as MRI and nuclear imaging also have no limit of penetration depth and can provide brain images in three dimension with better spatial resolution [59]. However, nuclear imaging is unsuitable for long-term monitoring, and the effects of MRI are sometimes impeded due to artifacts e.g., intracranial hemorrhage [62]. Moreover, for visualization purposes with medical imaging modalities (e.g., magnetic resonance imaging), cells can be labeled with contrast agents (e.g., iron oxide nanoparticles), which allows their identification from the surrounding environment. Despite the success of revealing cell biodistribution in vivo, most of the existing agents do not provide information about the status and function of cells following transplantation [54]. Consequently, fluorescence imaging might be an alternative or adjuvant

technique under some conditions, such as hemorrhagic infarction and traumatic brain injury.

However, the main problem of optical imaging is the difficulty in locating limited light penetration (from a few millimeters to centimeters); thus, it is not appropriate for large animals. Owing to the absorption and scattering of light, the fluorescence signal in deep tissue is frequently very weak, and the spatially resolved intensity is also low. Additionally, the depth of the light source in the animal model and the minimum observed cell number can vary. Therefore, tracking stem cell using optical imaging technology is only applicable to small animals and cannot be applied to whole body imaging of humans [55]. In spite of BLI that has already been used to study in vivo stem cell migration, viability, immunogenicity, and tumorigenicity in small animal studies, all luciferase emissions are in the visible spectrum, which is prone to scattering and absorption by the tissue. Even firefly luciferase, with a comparatively long peak wavelength of 562 nm, is limited for use in small animals due to a maximum penetration of 3 cm of tissue. Moreover, for BLI, the requirement of engineering cells runs the risk of introducing unwanted mutations. Therefore, BLI may not be feasible for clinical translation [63].

14.4.4 Conclusion and Future Perspectives

Stem cells are highly specialized cells with the ability of self-renewal and multidirectional differentiation potential. In recent years, the application of stem cell transplantation for the treatment of cerebral stroke is utilized increasingly. In order to achieve the perfect therapeutic effect after transplantation, it is necessary to monitor the transplanted cells. Transplanted stem cells can induce and enhance functional recovery in experimental stroke. Invasive analysis has been extensively used to provide detailed cellular and molecular characterization of the stroke pathology and engrafted stem cells. However, postmortem analysis is inappropriate in revealing the time scale of the dynamic interplay between the cell graft, ischemic lesion, and endogenous repair mechanisms [64]. The optical imaging techniques in vivo can trace the survival and migration of transplanted stem cells in real-time and provide a lot more important information. Toshiya Osanai et al. had successfully applied the in vivo fluorescence imaging for monitoring the migration and survival of transplanted stem cells in the brain in real-time [50]. Although this technology is still in its infancy, with the continuous development of medical technologies, these powerful new techniques of using ultrafast lasers, dual-photon imaging, and ballistic photon imaging may see progress in the areas of sensitivity, detection depth, and spatial resolution. In addition, these technologies would allow researchers to non-invasively obtain complete brain images in the future and to monitor transplanted stem cells in the central nervous system [57, 59].

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