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REVIEW

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Opportunities and Challenges: Stem Cell-Based Therapy for the Treatment of Ischemic Stroke

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SUMMARY

Stem cell-based therapy for ischemic stroke has been widely explored in animal models and provides strong evidence of benefits. In this review, we summarize the types of stem cells, various delivery routes, and tracking tools for stem cell therapy of ischemic stroke. MSCs, EPCs, and NSCs are the most explored cell types for ischemic stroke treatment. Although the mechanisms of stem cell-based therapies are not fully understood, the most possible functions of the transplanted cells are releasing growth factors and regulating microenvironment through paracrine mechanism. Clinical application of stem cell-based therapy is still in its infancy. The next decade of stem cell research in stroke field needs to focus on combining different stem cells and different imaging modalities to fully explore the potential of this therapeutic avenue: from bench to bedside and vice versa.

Stroke is a major cause of serious disability and the second cause of death in the world [1]. Stroke takes up 2–4% of total healthcare costs. With the advent of the aging era, the burden will continue to increase [2]. Currently, the only effective treatment, tissue plasminogen activator (tPA), is limitedly applied because of its narrow treatment window and relative high risk of hemorrhage. Growing evidence suggest that stem cells are potentially beneficial for neurological functional recovery following ischemic stroke. In this review, we discuss the critical issues in basic and clinical research regarding stem cells therapy in ischemic stroke.

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. Recently, there are also several studies that investigated the efficacy of transplantation of a combination of different stem cells.

Mesenchymal Stem Cells (MSCs)

The therapeutic potential of MSCs was studied extensively in the ischemic brain [3,4]. However, it was still unclear how the engrafted cells contributed to the functional recovery after ischemic stroke. It was recognized that MSCs exerted their beneficial effects mainly through immune-modulatory and paracrine mechanisms than through cell replacement, given its limited neuronal

differentiation capacity [5]. *In vitro* studies demonstrated that the conditional medium of MSCs significantly promoted neurite outgrowth of dorsal root ganglion [6]. When MSCs were cocultured with neurons exposed to glutamate, they significantly ameliorated the glutamate induced neuronal injury by releasing soluble neuroprotective factors [7]. *In vivo* studies also showed that injecting MSCs into rats following ischemic stroke could attenuate blood brain barrier (BBB) destruction and improve neurobehavioral recovery through inhibiting inflammation, inducing neurogenesis and angiogenesis [8]. MSCs treatment could also maintain the integrity of BBB by inhibiting aquaporin-4 upregulation [9]. Stereotactically transplanted MSCs markedly improved the recovery of glucose metabolism in the peri-infarct neocortex in an ^{18}F-fluorodeoxyglucose PET study [10]. MSCs transplantation also enhanced axonal plasticity, and interhemispheric and intracortical connections in stroke rats [11]. Recently, a number of studies showed that neurotrophic gene modification enhanced the therapeutic effects of MSCs. For example, intravenous injection of brain derived neurotrophic factor (BDNF) modified human MSCs, and/or combination of Angiopoietin-1 and vascular endothelial growth factor (VEGF) gene-modified human MSCs into ischemic rats yielded better therapeutic effects than nonmodified MSCs through promoting angiogenesis and neovascularization [12,13].

Neural Stem Cells (NSCs)

NSCs existed in the subventricular zone (SVZ) and subgranular zone (SGZ) in adult brain [14]. After ischemic stroke onset, endogenous NSCs could proliferate and migrate into the injured region, promoting tissue repair [15,16]. While ablating endogenous NPCs-expressing doublecortin (DCX) caused inhibition of neurogenesis and worsened outcome [17]. These data suggest that endogenous NSC contribute to postischemic stroke repair. However, the number of endogenous NSCs was insufficient for complementing lost neurons, and few NSCs were found to differentiate into neurons [18]. NSCs transplantation could enhance neurogenesis and is regarded as a promising therapy strategy for ischemic stroke [19,20]. Preclinical studies explored the feasibility of using NSCs to treat ischemic stroke. NSCs were found to survive and differentiate into neurons after transplantation, consequently, improve neurological function recovery in ischemic rodent [21,22]. Studies showed that delayed intravenous transplantation of NSCs at 3 days after ischemic stroke exhibited delayed neuroprotection by suppressing inflammation and focal glial scar formation, suggesting that NSCs had the potential to extend the therapeutic time window for ischemic stroke treatment [21]. VEGF- or Akt-1-modified NSCs also improved neurological function recovery after ischemic stroke by increasing focal angiogenesis and neuronal survival [23,24]. These experiments identified NSCs as an effective candidate for ischemic stroke treatment.

Vascular Progenitor Cells (VPCs)

VPCs were first isolated from ESCs and defined as ESC-ECs [25]. They could differentiate into endothelial cells or smooth muscle cells when they were induced with VEGF or platelet-derived growth factor-BB (PDGF-BB), respectively. Experimental studies showed that VPCs transplantation played a positive role in the vascular repairing and remodeling during ischemic diseases. In a mouse hind limb ischemic model, intravenously or intramuscularly transplanted VPCs integrated into endogenous blood vessels, significantly attenuated the ischemic injury [26,27]. Compared to human umbilical vein endothelial cells (HUVEC), transplanted VPCs exhibited better therapeutic effect in mouse model of myocardial infarction [28]. In addition, cotransplantation of neural precursor cells (NPCs) and VPCs into ischemic stroke rats resulted in better neurovascular recovery than transplantation of NPCs alone [29]. These results suggest that VPC is another promising candidate for treatment of ischemic stroke, especially for combinatory transplantation regimens.

Endothelial Progenitor Cells (EPCs)

In 1997, Asahara first isolated Flk-1$^+$/CD34$^+$ cells from human peripheral blood and found that these cells could integrate into blood vessels when they were transplanted into a hind limb ischemic mouse model [30]. EPCs were usually generated and maintained in bone marrow and could migrate into lesion region to participate in blood vessel remodeling and repair [31–33]. Recent studies showed that EPCs transplantation increased cerebral blood flow, reduced infarct volume, reduced neuronal cell death, induced focal angiogenesis and neurogenesis, and improved neurobehavioral recovery after ischemia [31,33–35]. Grafted EPCs could secret neurotrophic factors, which was supported by the evidence that EPCs medium could also promote angiogenesis [36] [37]. These results support that EPCs have great therapeutic potential for cerebral ischemia treatment, most possibly through both directly integrating into blood vessels and secreting paracrine trophic factors.

Embryonic Stem Cells (ESCs)

Embryonic stem cells have unlimited self-renewal capacity and are multipotent. However, their tumorigenic risk raised a safety concern as many studies reported that transplanted ESCs induced teratoma formation in receiving animals [38,39]. Therefore, transplantation of tissue-specific stem cells differentiated from ESCs might be a more promising choice for ischemic stroke therapy than undifferentiated ESCs. Study showed that ESCs-derived NPCs survived for up to 12 weeks after transplantation into ischemic rats, with 30% of them differentiated into neurons and 28% of the differentiated neurons exhibited electrophysiological activity [40]. This study strongly supported that ESCs-derived NPCs improved neurobehavioral recovery through neuronal replacement. In addition, transplantation of ESCs-derived NSCs into naive nude ischemic rats improved their ischemia-impaired forelimb recovery without inducing tumor formation [41]. These studies showed that ESCs-derived NPCs had a great potential for cerebral ischemia treatment. However, ethical controversy severely limited the clinical application of ESCs.

Induced Pluripotent Stem Cells (iPS)

iPS cells can be potentially generated from patient fibroblasts, which might help to overcome ethical concerns of NSCs and ESCs. Studies demonstrated that iPS cells could specifically differentiate

into glutamatergic neurons, motor neurons, and GABAergic neurons. These cells hold great promises for the treatment of various neurological diseases. Intracerebrally transplanted iPS cells were shown to migrate to the ischemic region and differentiate into neurons, reduce the infarct volume, and improve neurobehavioral recovery in rats [42,43]. However, iPSs have also been reported to induce teratoma formation although a great number of neuroblasts and a few mature neurons emerged at the same time in the ischemic region [44]. Recently, a novel strategy was reported that using a combination of Oct4 and Sox2 plasmid transfection with hypoxia conditioning to generate iPS cells (iPS-OSH cells). iPS-OSH-derived NPCs differentiated into neurons and astrocytes after being injected into ischemic mice brain, accompanied by improved neurobehavioral recovery [45].

A number of studies showed that iPS-derived NSCs were safer and more effective than iPS cells for the treatment of cerebral ischemia. A recent study showed that iPS-derived NSCs exhibited cortical phenotype and electrophysiological property of mature neurons with evidence of integration into host circuitry in mouse, without tumor formation [46]. It was also reported that iPS-derived NSCs could migrate into the peri-focal region and differentiate into neurons and astrocytes, consequently, improve neurological function recovery of a rat model [47]. These data suggest that iPS-derived NSCs have great potential to improve neurological functional recovery after ischemic stroke.

Challenges of Stem Cell Treatment for Ischemic Stroke

Stem cell transplantation showed promising results for regenerating lost tissues. However, several challenges remain to be overcome before stem cell therapy can be successfully applied to ischemic stroke treatment. First, the low survival of transplanted stem cells in injured area reduced efficacy of stem cell therapy [48]. Such challenge is faced by stem cell therapy applications in the treatment of other diseases as well. <1% MSCs were detected in the ischemic kidney 1 h after intravenous injection in rats. The number of MSCs continuously decreased in the following days and the remaining MSCs did not replace renal epithelial cells. Direct injection of MSCs into ischemia kidney also exhibited poor cells survival and did not contribute to renal structural repair [49]. A study focused on myocardium infarction in mice showed that only 1% MSCs infused in infarct area 4 h after transplantation [50]. Direct grafting cardiomyocytes into infract heart showed low cell viability. 32% of transplanted cells became TUNEL-positive apoptotic cells within 1 to 4 days posttransplantation [51]. Local injection of MSCs showed continuously decreased engraftment of 27% at 7 days, 7.6% at 14 days, and 2.5% at 28 days, although transplanted MSCs accelerated wound closure [52]. In a model of ischemic stroke, only 0.3% NSCs transplanted via intravenous injection accumulated in the brain 3 days posttransplantation. Interestingly, NSCs transplantation still exhibited beneficial therapeutic effects, including reduced neuronal degeneration and brain atrophy as well as improved motor coordination, although few NSCs emerged in the brain [19]. A recent meta-analysis showed that MSCs transplantation for the treatment of ischemic heart could improve left ventricular ejection fraction (LVEF), reduce infarct size, lower low voltage (LV) end-systolic volume, and LV end-diastolic volume [53]. The dose of transplanted MSCs was associated with LVEF improvement after MSCs injection in acute myocardial infarction [54]. The study found that a minimum of 4×10^7 cells was needed to achieve good outcomes based on MSCs transplantation in patients [53]. Collectively, stem cell transplantation faced a challenge of low cell survival in the injured area, eventually limiting stem cells beneficial effects.

The second challenge is to improve the migration of transplanted cells into the lesion region and integration into the host system. However, many recent studies suggest that integration of transplanted stem cells might not be fully necessary for them to exert their beneficial effect. The third challenge of translating stem cell therapy from the bench to bedside is the lack of knowledge regarding the fate of transplanted cells. Even though many studies have shown that intravenous and intra-arterial injection of stem cells can be beneficial for ischemic stroke recovery, little information is available regarding to where these cells migrated and what they differentiate into. Whole-body live imaging tools need to be developed for tracking transplanted cells and assessing their bioavailability and distribution.

Current Strategies to Improve the Efficacy of Stem Cell Therapy

Most of the efforts put to improve efficacy were spent on improving cell survival and homing to lesion sites. Preconditioning and gene modification methods were shown to be effective in improving the survival of transplanted stem cells. Chemokine, cytokine, and growth factor pathways were explored to improve the homing of transplanted cells.

Strategies to Enhance Survival of Transplanted Stem Cells

Damaged tissue and microenvironment affected stem cells survival, migration, and differentiation [55,56]. Hypoxic preconditioning was one of effective approaches to enhance stem cell survival in ischemic environment. Transplantation of hypoxia preconditioned ESCs-derived NPCs to the mouse brain after ischemic stroke reduced cell death by 30%–40%, enhanced NPCs differentiation, and accelerated functional recovery [57]. MSCs under hypoxia preconditioning exhibited enhanced cell survival, neuronal differentiation, and regenerative capability after ischemic stroke in rats [58]. Oxidative stress, heat shock, and BDNF pretreatment also enhanced stem cells survival *in vitro* or *in vivo*. Exposing NPCs in a noncytotoxic dose (0.5–5 micromolar) of hydrogen peroxide (H_2O_2) enhanced NPCs survival under lethal dose of H_2O_2 condition [59]. H_2O_2 pretreatment enhanced the survival of transplanted MSCs in mice with myocardial infarction, accompanied by better therapeutic effects [60]. Heat shock was reported to increase Sca1+ stem cells survival in ischemic heart [61]. BDNF pretreatment enhanced NSCs survival at the brain cortex in hypoxia–ischemia mice [62]. In addition, gene modification was another promising strategy to enhance transplanted stem cells survival. Intrastriatal injection of TAT-heat shock protein 70 (Hsp70) transduced NSCs after ischemic stroke in mice enhanced transplanted NSCs survival and improved functional recovery [63]. Heat-shock protein 27 (Hsp27) modification reduced MSCs

apoptosis when transplanted in myocardium infarction and improved ischemic heart function [64]. Recent study showed that a novel gene delivery system of facial amphipathic bile acid-pHI-VEGF could induce VEGF overexpression in MSCs, which enhanced MSCs proliferation in normoxia or in hypoxia *in vitro*. Transplanting VEGF-modified MSCs promoted capillary formation in the infarct region and reduced left ventricular remodeling [65]. Protein kinase B (Akt) and hemeoxygenase-1 (HO-1) gene-modified EPCs promoted EPCs migration, cell survival, and neovascularization after myocardial infarction in nude mice [66]. Using bioengineered scaffolds to mimic the physiological environment suitable for survival of stem cells is another strategy [67]. Scaffolds could protect stem cells from mechanical damage during the process of injection and significantly improve cells viability [68]. Transplanted MSCs seeded in carbohydrate-based hydrogel scaffolds improved stem cells survival, promoted focal angiogenesis, and accelerated wound healing [69]. BDNF-modified scaffolds enhanced cortical NSCs proliferation and promoted NSCs differentiantion into neuronal cells and oligodendrocyte *in vitro* [70]. Recent study showed that ES cell-derived progenitor motor neurons could survive in 3D fibrin scaffolds and differentiate into neurons, oligodendrocytes, and astrocytes after transplantation to a mouse model of subacute spinal cord injury [71]. In conclusion, these results suggested that the bioengineered scaffold could be a valuable tool for enhancing stem cell survival.

Strategies to Enhance Stem Cells Homing and Migration

MSCs transplantation showed a promising future in the treatment of many diseases [72]. However, only 1% MSCs direct homing to the injured area [50]. Many approaches including hypoxic preconditioning [73,74], primed with valproate and lithium [75], cytokine cocktail pretreatment [76], and viral-mediated CXCR4 transduction [77] were used to enhance MSCs survival, homing, and migration. SDF-1a/CXCR4/CXCR7 played an important role in MSCs survival and migration both *in vitro* and *in vivo* [78]. Using lent-viral vector transduction to enhance CXCR4 expression in MSCs promoted MSCs migration via activating Akt signaling pathway. CXCR4 could increase cytokines and growth factors release in injured brain after MSCs transplantation, contributing to better functional recovery [72]. Novel chemical DMPE-PEGs was used to link CXCR4 on the surface of MSCs and reduce the process time for CXCR4 overexpression in MSCs *in vitro*. DMPE-PEGs-CXCR4-modified MSCs could migrate toward an SDF-1a gradient and exhibit higher viability and proliferation ability [79]. The new method seemed to be helpful for improving MSC treatment efficacy for various diseases.

SDF-1a/CXCR4/CXCR7 signal pathway was the most common mechanism of modulating NSCs survival, homing, and migration. NSCs could migrate toward ischemic region, where SDF-1a was upregulated. SDF-1a promoted NSCs proliferation and migration when added into NSCs culture medium [80]. CXCR4 was essential for SDF-1a-mediated NSCs migration, while CXCR7 was essential for NSCs survival [81,82]. In CXCR4-deficient mice, NPCs migration decreased [83]. In CXCR7 knockout mice, NPCs apoptosis increased [82]. Other cytokines/chemokines or molecular such as monocyte chemoattractant protein-1 (MCP-1, CCL-2), growth-regulated oncogene-alpha (GRO-a), nitric oxide (NO), erythropoietin (EPO), and VEGF were also involved in directing NSCs migration after ischemic stroke [84–86]. VEGF promoted NPCs migration via activating VEGFR-2 *in vitro*, which required the involvement of fibroblast growth factor 2 (FGF-2) [87]. Injection of VEGF to the forebrain in nude mice promoted the migration of transplanted NSCs from contralateral region into the VEGF-injected hemisphere [88]. EPO, which was a part of inducible cytokines respond to hypoxia condition, was reported to act on NSCs via NF-κB translocation. Injecting EPO to adult mouse lateral ventricle decreased the number of NSCs in SVZ, but increased the number of new interneurons in olfactory bulb (OB), indicating that EPO promoted NPCs migration into the OB [89]. Despite the many discoveries of molecular mechanisms mediating transplanted NSCs homing and migration, strategies on enhancing NSCs homing and migration seemed to be insufficient, possibly owning to the hostile environment caused by hypoxia. Future study should pay more attention on enhancing NSCs homing and migration to improve NSCs-based therapy in clinic application.

Blocking SDF-1a/CXCR4 pathway by AMD3100-inhibited endogenous EPCs mobilization after ischemic stroke in rats, causing lower capillary formation, reduced cerebral blood flow and worsened neurological function [90]. Circadian gene period2 (per2) improved EPCs tube formation and migration ability *in vitro*. Per2 deficiency mice showed larger infarct size, worse cardiac function, and lower numbers of CD34+ EPCs and capillary density in myocardium 4 weeks after myocardial infarction [91]. A study showed that parathyroid hormone (PTH) administration after ischemic stroke in mice enhanced the number of circulating CD34+/Flk+ EPCs, promoted the migration of endogenous neuroblasts toward the peri-infarct region, and improved functional recovery [92]. Conversely, in a model of oxygen-induced retinopathy (OIR), peroxisome proliferator-activated receptor alpha (PPARα) suppressed EPCs mobilization and homing toward retinal by inhibiting HIF1-a/SDF-1a pathway, causing low neovascularization [93]. In conclusion, enhancing the migration and homing toward the injured region of transplanted EPCs would help it exert regenerative effects. Further study is needed to illustrate the molecular mechanism of EPCs migration and homing. Strategies for enhancing EPCs direct homing and migration is beneficial for EPCs-based therapy.

Combination Therapy to Improve Stem Cell Efficacy

In addition to improving the survival of stem cells and their migration into the lesion area, other combination therapy has been explored to improve the efficacy of stem cell therapy. A recently study reported using optogenetically engineered NSCs for transplantation. Chronic optogenetic stimulation of these transplanted cells resulted in enhanced sensorimotor performance in rat ischemic stroke model [94].

Stem Cell Tracking

Evaluations of the effect of stem cell-based therapy for ischemic stroke were usually based on a reduction of infarct volume or improvement on neurobehavioral outcomes. The difficulty of

assessing and tracking administered stem cells noninvasively in real-time hindered the development of stem cell-based therapy in ischemic stroke. Novel imaging modalities are urgently needed to help optimize stem cell transplantation protocols and promote their translation. In response to this need, various strategies for stem cell tracking were explored in recent years.

Magnetic Resonance Imaging (MRI)

MRI is one of the most promising and well-studied noninvasive imaging modalities for stem cell tracking. To track transplanted stem cells by MRI, stem cells need to be labeled with contrast agents to gain enough difference in signal intensity. In early studies, gadolinium rhodamine dextran (GRID) was used to track NSCs in the ischemic brain, demonstrating the feasibility of using MRI to monitor NSCs migration. Superparamagnetic iron oxide (SPIO) nanoparticle became the first choice for stem cell labeling [95]. SPIO was more sensitive and safer than other contrast agents as SPIO could be degraded by physiological metabolism and was already approved by FDA [96]. SPIOs provided high spatial resolution but limited sensitivity in MRI, which could detect irons at concentrations from micromolar to millimolar range [97]. Yang and colleagues extensively explored the feasibility and biocompatibility of tracking SPIO labeled stem cells by MRI [98,99]. They transplanted high MR sensitivity fluorescent-magnetite-nanocluster (FMNC)-labeled MSCs into the contralateral hemisphere in the ischemic mice brain and subsequently found that FMNC-labeled MSCs could migrate toward the peri-focal region of the ipsilateral hemisphere through corpus callosum, as detected by MRI and fluorescent imaging [98]. They also intracerebrally injected fluorescent mesoporous silica coated SPIO (fmSiO4@SPIONs)-labeled NPCs into the ischemic mouse brain and examined the brain with MRI and histological method [99]. These two studies suggested that fluorescent-modified SPIO allowed highly effective tracking of stem cells and held great promise for MRI application. However, the limitation of SPIO labeling was also highlighted recently. The specificity of MR signal was firstly questioned. Histological analysis revealed only iron-containing macrophages at the injection site, but very few viable stem cells could be detected at 3 weeks after stem cells delivery [100]. At least one study reported that Feridex was detected even after the death of grafted Feridex-labeled cardiomyoblasts in rat myocardium, suggesting that there could be mismatches between MRI signal and the stem cells of interest [101]. Secondly, the SPIO concentration was halved with cell divides, causing signal decrease, which made it challenging for long-term tracking, especially when the cells were grafted to environment that promoted rapid cell proliferation [102].

Overall, MRI has several advantages as a noninvasive approach to monitor transplanted stem cells. Future studies are still needed to address specificity and sensitivity issues to accelerate its clinical application.

Optical Imaging

Optical imaging techniques included fluorescence imaging, quantum dots imaging, and bioluminescence imaging (BLI). These methods required the introduction of a fluorescent label in the forms of fluorescent proteins, such as green fluorescent protein (GFP), or fluorescent dyes, such as DiD, to stem cells [103–105]. Fluorescent dyes and fluorochromes had limited half-life and were diluted during cell division, making them not optimal for long-term tracking. BLI was the most utilized optical imaging modality owing to its simplicity, accuracy, and quantitative capacity [106]. After transplantation of stem cells modified with a firefly or Renilla luciferase (Luc) enzyme, systemic injection of the luciferase substrates, D-luciferin, or coalenterazine allowed BLI to examine and quantify Luc-expressing cells [107].

BLI was widely used to track the migration and survival of transplanted NSCs in animal models for ischemic stroke. Combined with histological analysis, BLI was used to monitor the fate of grafted NSCs in the ischemic rat brain [108]. With BLI, NSCs were detected in a transient ischemia model in mice [109]. These studies suggest that BLI is a reliable noninvasive method with high sensitivity for the long-term monitoring of transplanted stem cells in stroke models. However, the spatial resolution and the penetration depth of BLI were limited, which made this method unsuitable for clinical application at current stage.

SPECT/PET Imaging

SPECT and PET were routinely used for both clinical diagnosis and monitoring the effect of postischemic therapy and had already been employed to track radioactive nuclides-labeled stem cells for many years. In a pioneering study, ^{111}In-oxine-labeled EPCs were found to be able to migrate to injured rat myocardium after intravenous injection using SPECT imaging [110]. A minimum number of 10,000 ^{111}In-tropolone-labeled MSCs could be detected by SPECT imaging [111]. After intravenous injection into farm pigs, ^{111}In-oxine-labeled MSCs were mainly found in the lung [112]. Recently, a debris impulse response function (DIRF) could be used to calculate the contribution of extracellular ^{111}In in the canine myocardium to assess radiolabel leakage after the death of transplanted cells [113]. After direct epicardial injection, a series of SPECT images were captured to measure the time-dependent radiolabel clearance.

The most commonly used radiolabeling probe for PET imaging was 18F-fluorodeoxyglucose ([18F]-FDG), which was approved by the FDA. Several studies reported direct imaging of transplanted cells with 18F-FDG [114,115]. In a clinical study, 18F-FDG-labeled human bone marrow cells were monitored by PET to determine the distribution of intracoronary infused cells, only 1.3% to 2.6% of BMCs were detected in the infarct myocardium, while the remaining activity were mainly found in the liver and spleen [116]. Another strategy for cell tracking with PET was to use enzymatic reactions that could immobilize radiolabeled substrates. The herpes simplex virus type 1-derived thymidine kinase (HSV1-tk), which could exclusively phosphorylate substrates composed of acycloguanosines, was routinely used to label stem cells. HSV-tk-modified stem cells were tracked in the rodent brain, human ESCs, and C17.2 NSCs [117,118]. It was important to note that 18F-FHBG could not be used to detect labeled cells in the normal brain because its substrate could not cross intact BBB [118]. Nonetheless, this method was a promising candidate for tracking stem cells in injured brain, in which BBB was compromised in the acute phase.

It should be noted that any single aforementioned imaging modality has its advantages and drawbacks. Combining several imaging modalities for stem cell tracking is the current trend to solve the problem. MRI/SPECT (PET) and BLI/PET dual-mode imaging have been pursued to track stem cells *in vivo*. Cao et al. monitored the survival, migration, and proliferation of ESCs with a novel triple-fusion reporter gene that consisted of firefly luciferase, monomeric red fluorescence protein, and truncated thymidine kinase using BLI and 18F-FHBG PET [119]. Another recent study demonstrated the feasibility of using multimodal imaging to monitor the fate of grafted MSCs into the rodent brain [120]. BLI had higher sensitivity for detecting Luc-expressing cells, especially if BBB was intact. 18F-FHBG-PET was useful for monitoring TK-expressing cells in conditions in which BBB was disrupted, while MRI after gadolinium diethylenetriamine pentaacetic acid (Gd-DTPA) administration can be used for evaluating the BBB integrity [118]. It is clear that developing safer, more effective, and accurate imaging modalities is crucial to advance our understanding of stem cell-based therapy.

Routes of Transplantation

Neurobehavioral outcomes after ischemic stroke were observed with intracerebral, intraventricular, intravenous, and intra-artery deliveries of stem cells [121]. However, the most optimal delivery route for specific cell types is unclear. Intracerebral injection induced cells to a chosen location and had the highest cell retention in the brain compared to intra-arterial and intravenous delivery [122], but it could have higher risks for clinical translation due to its invasive procedure. An early clinical trial indicated that intraparenchymal cells implantation caused severe adverse events involving seizures and transient motor function impairment [123]. Additionally, large number of grafted cells retained in the brain raised the possibility of tumor formation, especially when ESCs and iPSs were used for transplantation [124]. In contrast, intraventricular transplantation was less invasive and no stem cell-related tumor formation was observed to date. Intraventricular transplantation of amniotic fluid-derived stem cells significantly improved short-term memory, motor coordination, sensorimotor ability, and somatosensory functions after ischemic stroke [125]. However, intraventricularly injected human NSCs into ischemic rat brain did not show improvement [126]. The safety and efficiency of locally delivering stem cells for stroke need to be explored in further study.

Intravenous delivery was safer and more feasible. Intravenously transplanted stem cells have the potential to secret angiogenic and immunomodulatory factors to the whole body [50]. Recent studies show that one can enhance stem cells homing by labeling the grafted cells with SPIO and guide with exogenous magnetic field. Riegler et al. magnetically targeted intravenously injected SPIO-labeled MSCs by a clinically applicable permanent magnet, which resulted in a 6-fold increase in cell retention following balloon angioplasty in a rabbit model, associated with a decrease in restenosis 3 weeks after cell delivery [127]. The homing of SPIO-labeled EPCs was greatly increased in ischemic hemisphere with magnetic field treatment in stroke mice, accompanied with improved neurobehavioral recovery, attenuated atrophic volume, and increased angiogenesis and VEGF expression [128].

Intra-arterial administration contributed to more cells retaining in the brain than intravenous delivery and was beneficial for behavioral recovery [129]. However, intra-arterial transplantation also resulted in high mortality (about 40%), high blood flow reduction (up to 80%), and a significant morbidity, potentially due to cell accumulation and microemboli, especially when large-sized stem cells (e.g., MSCs) were transplanted intra-arterially into ischemic animals [130]. It was demonstrated that cell size, cell dose, and infusion velocity were associated with blood flow reduction and morbidity. Infusion velocity over 1 mL/min could cause microstroke, whereas a low velocity of 0.2 mL/min was safe (e.g., glial-restricted precursors, diameter<15 μm). Infusion of 2×10^6 MSCs (diameter = 25 μm) caused a profound decrease of cerebral blood flow [131], but 1×10^5MSCs did not compromise MCA flow, and intra-arterial transplantation of 1×10^5 MSCs achieved the same therapeutic effects as intravenous delivery of 1×10^6 MSCs [130]. To reduce the risk of reduction in cerebral blood flow and micro-hemorrhage after intra-arterial injection, several strategies were already developed, including a microneedle injection technique to avoid the development of microstroke [132]. Another study suggested that intra-arterial injection of 1×10^6 MSCs derived from 3D spheroids resulted in engraftment of the cells into the lesion and reduction of infarct volume along with restoration of neurologic function, without causing vascular obstruction [133]. Taken together, current data suggested that extra caution should be taken when transplanting stem cells intra-arterially.

Intranasal administration is a noninvasive and alternative route for the delivery of stem cells into the brain. In normal mice, MSCs or glia cells delivered by intranasal administration could bypass BBB and migrate to brain parenchyma and cerebrospinal fluid (CSF) [134]. In transgenic mice of Parkinson's disease (PD) and Alzheimer's disease (AD) models, MSCs were detected in the olfactory bulb (OB), cortex, amygdala, striatum, hippocampus, cerebellum, and brainstem after intranasal delivery, with majority of the MSCs found in the OB and the brainstem [135]. Although the mechanism of intranasal delivery was unclear, olfactory nerve pathways, trigeminal nerve pathways, vascular pathways, and lymphatic pathways were possibly involved [136]. The safeness and efficiency of intranasal administration in various neurological disorders including ischemic stroke [73], hypoxia–ischemia [137], experimental autoimmune encephalomyelitis (EAE) [135], AD, and PD [138,139] were investigated. Currently, no side effects observed in stem cell transplantation via intranasal administration. MSCs were used in studies mentioned above for intranasal delivery. Whether intranasal administration was safe and effective for other stem cells delivery such as NSCs, EPCs, and ESCs needed to be further tested [136].

Mechanism of Stem Cell Therapy in Stroke

The initial goal of using stem cells to treat ischemic stroke was to regenerate the stroke-damaged tissue by cellular replacement. Quantitative studies showed that about 1/3 of locally injected cells migrated to the ischemic area under the action of chemokines [140], while very few intravenously injected cells arrived to the lesion area [141]. Surprisingly, many researchers found that 80%

of transplanted cells died at 3 days after transplantation, due to the hostile microenvironment of the lesion site [142]. What was the function of the remaining survival cells in the ischemic region? Different cell types might have different functions. In various investigations, about 20–60% of NSCs [22,143,144], 40%–66% of iPS [145,146], 30% of ESCs [40], and <2% MSCs [147] expressed neuronal markers. In our experience, when NSCs were transplanted into ischemic adult and aged rats brain, only 20% differentiated into Tuj-1$^+$ neurons, over 75% differentiated into GFAP$^+$ astrocytes [148]. Recent studies further explored the electrophysiological property of differentiated neurons by voltage-gated sodium currents. ESCs-derived precursors transplantation into ischemic rat brain 7 weeks after injection exerted spontaneous excitatory postsynaptic currents [40]. Neuroepithelial-like stem cells, which were generated from adult human fibroblast-derived iPS cells, could differentiate into mature neurons, exhibit electrophysiological properties, and receive synaptic input from host neurons, even send axonal projections to the globus pallidus [145]. This was the first evidence that iPS-derived stem cells could repair the injured brain through neuronal replacement. Evidence showed that cortical-specific iPS-derived neural progenitors exhibited electrophysiological properties of mature neurons with integration into host circuitry [46]. Until now, there is no clear evidence of MSCs differentiated into mature neurons with electrophysiological properties in stroke models.

A moderate to severe stroke model could cause over 75% of neurons to die [149], thus it was difficult to attribute stem cells mediated neurobehavioral recovery entirely to cellular replacement as only a small number of neurons were replaced. It was reasonable to explore another possible role of stem cells in the therapy, a bystander effect, referring to the fact that these grafted stem cells could directly release growth and trophic factors, or promote the release of such factors from host brain cells [150]. It was reported that systemically injected human umbilical cord blood cells into ischemic rats reduced cerebral infarct, improved behavioral recovery, and increased BDNF expression even though no cells could be detected in the brain [151]. Other studies reported that conditioned medium from stem cells could protect brain from ischemic injury [152–154]. Trophic factors, for example, VEGF or bFGF, had been implicated in stem cells mediated protection against ischemia and played an important role in angiogenesis and neurogenesis [151,155].

Human umbilical tissue-derived cells and bone marrow mononuclear cells could increase proliferation of endothelial cells and NSCs/NPCs by secreting angiogenic cytokines 2 weeks after cerebral ischemia [156,157]. We and other studies demonstrated MSCs treatment increased VEGF expression and angiogenesis in ischemic rats brain [158,159]. In addition, *in vitro* studies showed that VEGF increased in the conditioned medium of MSCs [160]. Further studies showed that angiogenic gene-modified stem cells had better angiogenic and therapeutic effects for stroke [13, 161–163].

The interaction between stem cells and innate / adaptive immune cells was also explored. It was suggested that the cross talk between immune cells and grafted stem cells determined therapeutic efficacy. Many studies had shown that NSCs [151,164], MSCs [165,166], iPS cells [167], bone marrow mononuclear cells [168], and umbilical cord matrix cells [169] attenuated inflammation after cerebral ischemia. It was conceived that grafted NSCs, although remaining undifferentiated, could secret immunomodulatory molecules incharge of enhancing tissue repair [170]. It was worth noting that immunomodulation by grafted cells might be independent of differentiation. In a mouse hemorrhagic stroke model, intravenously injected NSCs were found to accumulate in the spleen and attenuate the brain injury through inhibiting the splenic inflammation, whereas splenectomy eliminated the protective effects afforded by NSCs transplantation [164]. An interesting study *in vitro* demonstrated that human fetal NSCs continuously expressed immune-related genes and attenuated T lymphocyte proliferation and dendritic cells maturation [171].

Inflammatory mediators also influenced the function and fate of grafted stem cells. Primarily proinflammatory cytokines such as TNF-α, IL-1β, IFN-γ hindered the proliferation of injected NSCs by downregulating SVZ cell cycle [172]. ESCs-derived NSCs implantation activated innate immune cells and lymphocytes in the normal mouse brain, further suppressed neuronal differentiation through releasing IL- 6 [173].

Current Clinical Trials of Stem Cell Treatment of Ischemic Stroke

Currently, over 70 studies with known status are recorded at the clinicaltrials.gov website. About half of these studies have passed the phase I safety evaluation and entered phase II efficacy test. MSCs are the Star stem cells for clinical studies. A large number of clinical trials provided evidence that intravenous injection of MSC is safe and feasible in humans [174–176]. In a clinical trial study, intravenously transplanting 1×10^8 MSCs into five stroke patients showed functional recovery with 5-year follow-up [177]. Similarly, intravenously injected autologous serum cultured MSCs into 12 ischemic patients greatly reduced infarct volume and neurobehavioral deficits after 1 week of MSCs transplantation, without teratoma formation in 1-year follow-up [175]. However, scientific conclusions from these trials still need to be further demonstrated regarding to the lack of randomized study design, appropriate control group, and small sample size.

In contrast to MSCs, other cell types, such as NPCs, have been less frequently studied in clinical studies. Two clinical trials have demonstrated that injection of neuronal cells derived from a teratocarcinoma cell line is beneficial for stroke patients [178,179]. However, the sample size (4–7 patients per group) was too small to evaluate the therapeutic efficacy of NPC.

Although most of clinical studies achieved promising data, it's noted that one trial was terminated after intracerebral transplantation of fetal porcine NPCs into five patients in light of significant side effects were observed in two patients [123]. Another study demonstrated that intrathecal administration of cell suspensions from immature nervous and hematopoietic tissues has no side effects in 10 patients over 6-month observation [180]. Conclusions regarding the safety and efficacy of stem cell treatment in stroke patients could only be made unless large sample size and appropriate control studies are performed.

Summary

Stem cell-based therapies for ischemic stroke are under intensive investigation in experimental stroke animal model with many evidence of benefits. Much knowledge has been gained from these studies and early clinical trials, which investigated the safety and feasibility of cellular therapy for the cerebral ischemia. However, stem cell-based therapy is in its infancy. The mechanism of these strategies is not completely elucidated, and there are many hurdles that need to be overcome before clinical application. We do not know which stem cell and delivery route is the most effective for the cellular therapy, how many cells should be transplanted, and where these cells travel and what these cells eventually become, if they do survive. Each stem cell types and imaging modality has its own advantages and drawbacks. Cells with a readily available autologous source certainly hold more promise in clinical translation. Strategies to improve cell survival and enhance efficacy would strengthen the application potential of stem cell therapy. Further understanding of the mechanisms by which the stem cells exert their beneficial effect could potentially revolutionize the field. The next decade of stem cell research in stroke need to focus on combination of different stem cells and different imaging modalities for the stem cell therapy, to fully explore the potential of this therapeutic avenue.

Conflict of Interest

The authors declare no conflict of interest.

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