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Stem Cell-Based Therapies for Ischemic Stroke: Preclinical Results and the Potential of Imaging-Assisted Evaluation of Donor Cell Fate and Mechanisms of Brain Regeneration

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Abstract: Stroke is the second most common cause of death and is a major cause of permanent disability. Given the current demographic trend of an ageing population and associated increased risk, the prevalence of and socioeconomic burden caused by stroke will continue to rise. Current therapies are unable to sufficiently ameliorate the disease outcome and are not applicable to all patients. Therefore, strategies such as cell-based therapies with mesenchymal stem cell (MSC) or induced pluripotent stem cell (iPSC) pave the way for new treatment options for stroke. These cells showed great preclinical promise despite the fact that the precise mechanism of action and the optimal administration route are unknown. To gain dynamic insights into the underlying repair processes after stem cell engraftment, noninvasive imaging modalities were developed to provide detailed spatial and functional information on the donor cell fate and host microenvironment. This review will focus on MSCs and iPSCs as types of widely used stem cell sources in current (bio)medical research and compare their efficacy and potential to ameliorate the disease outcome in animal stroke models. In addition, novel noninvasive imaging strategies allowing temporospatial in vivo tracking of transplanted cells and coinciding evaluation of neuronal repair following stroke will be discussed.

Key words: ischemic stroke; mesenchymal stem cells; induced pluripotent stem cells; mechanisms of stem cell therapy; noninvasive imaging

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1. INTRODUCTION

Worldwide, stroke is the second single most common cause of death, accounting for 10–15% of deaths each year.[1,2] Moreover, stroke is an important cause of adult disability as 90% of patients that survive from a stroke are left with a residual deficit.[3,4] It might therefore be clear that stroke-related public and insurance costs constitute a major burden on healthcare systems worldwide.[1,2] Combining the expectation that the amount of people over the age of 65 will double by 2030, and that the risk of suffering a stroke doubles for each decade over the age of 55, will even lead to a further increase in patient numbers with permanent disabilities and socioeconomic burden.[2,4–7]

Despite this increased incidence, current available therapies are unable to sufficiently ameliorate the disease outcome or are even not applicable for subgroups of patients due to many contraindications as will be discussed below. Therefore, new therapeutic strategies are needed for treating and preventing stroke that can be applied to patients with distinct risk profiles and in a broader time frame, as time plays a crucial role in the treatment of acute ischemic stroke. In addition to clinical advances in stroke management, cell-based therapies have emerged as a potential candidate to promote functional recovery in patients suffering from stroke.[8] Despite the promising results achieved with cell-based therapies in stroke, the host response, the precise mechanisms of action of these therapies, and the fate of the donor cells remain largely unknown.[9] Therefore, noninvasive imaging modalities have been developed that are able to provide detailed temporospatial and functional information on the donor cell fate, the host microenvironment, and endogenous repair mechanisms,[10] which will be discussed later.

A. Pathophysiology of Stroke

The pathophysiology of stroke can be defined as a neurologic dysfunction of vascular origin with the sudden or rapid occurrence of symptoms and signs corresponding to the involvement of focal areas in the brain.[11] Two different types of stroke can occur: ischemic stroke (80–85%) and hemorrhagic stroke (15–20%). Ischemic stroke is most frequently caused by thromboembolisms while hemorrhagic stroke most often results from vessel wall pathology associated with hypertension and microaneurysms.[12] This review will only focus on ischemic stroke as the main pathology.

In ischemic stroke, the blood supply to certain brain areas is compromised due to vascular occlusion thereby causing several changes at the (sub)cellular level and ultimately tissue damage. These cellular and molecular processes start with energy depletion followed by glutamate release leading to glutamate-induced excitotoxicity, ion channel dysfunction, and free radical production. These processes in turn disrupt the cellular membrane, damage mitochondria and DNA, generate an immune response, and trigger necrotic and apoptotic cell death (Fig. 1).[13] In the ischemic core, these cellular changes are irreversible.[14] However, the tissue surrounding the core, also termed as the ischemic penumbra, is functionally impaired but still viable.[15] This area "at risk" is therefore considered as the main target for therapeutic interventions that are believed to exert a protective effect in intervening with the cellular processes discussed above.[16,17] Using noninvasive imaging methods, the ischemic penumbra has been divided in additional border zones characterized by different grades of hypoperfusion and varying risk of progressing toward lost infarcted tissue if a proper treatment is not initiated (Fig. 1).[18,19]

When considering therapies that are aimed to salvage the ischemic penumbra by restoring perfusion, it is also important to take into account that restoring the blood flow in ischemic tissue by thrombolytic treatment can lead to secondary damage by reperfusion injury.[13,20] This reperfusion injury is mediated by leukocyte infiltration through local disruption of the blood–brain barrier (BBB) and accompanying matrix metalloprotease (MMP) production in

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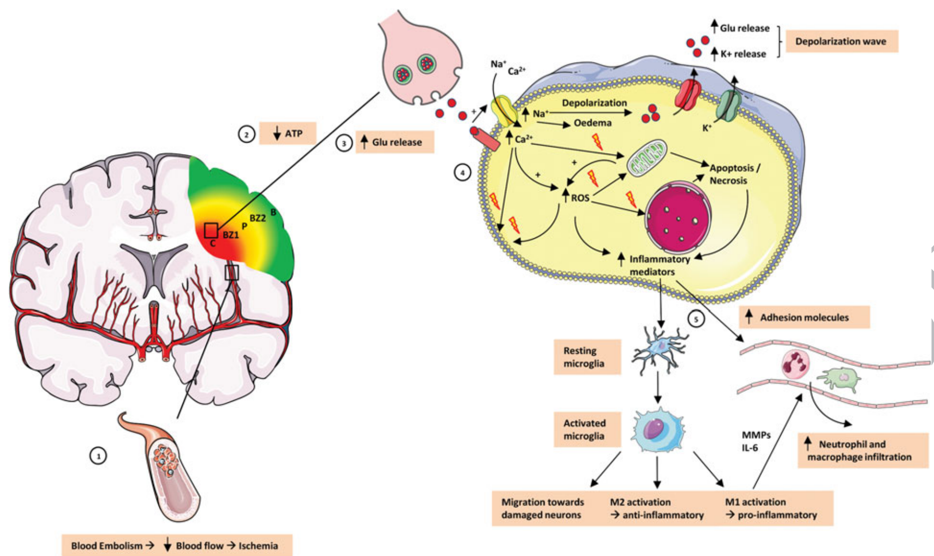

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Figure 1. Areas at risk and pathophysiology of ischemic stroke. (1) Blood flow to focal areas of the brain is diminished by vascular occlusion by, for example, an embolism. The affected ischemic tissue can be divided into the ischemic core (C) where tissue damage is irreversible, the salvageable ischemic penumbra (P), and a zone of benign oligemia (B) where blood supply can be obtained by leptomeningeal collaterals. Additional border zones with different grades of hypoperfusion and varying risk of progressing toward unsalvageable tissue if a treatment is not initiated were identified with perfusion-weighted MRI. These areas are the core-penumbra border zone (BZ1) and the penumbra-benign oligemia zone (BZ2). (2) The cellular changes ultimately leading to cell death initiate with ATP depletion due to ischemia, followed by depolarization of the affected neurons that triggers (3) glutamate release. (4) Glutamate-induced excitotoxicity is mediated by an elevated sodium and calcium influx that causes cell swelling, a depolarization wave that will lead to damage in neighboring cells, activation of a cascade of enzymatic reactions ultimately leading to membrane and mitochondrial damage and ROS production, which will additionally damage mitochondria and DNA ultimately leading to cell death. (5) Necrotic/apoptotic neurons secrete inflammatory mediators that activate resting microglia and enhance neutrophil and macrophage infiltration. The effects of activated microglia vary and include migration toward and phagocytosis of damaged neurons and depending on the M1/M2 activation state of activated microglia, proinflammatory and/or anti-inflammatory mediators are released. Image was created using Servier Medical Art.

addition to stimulation of reactive oxygen species (ROS) production, thereby damaging the reperfused environment.[13,14,20–22] In turn, the reperfused ischemic stroke lesion can transform into a petechial hemorrhage that does not influence the prognosis or it can transform into an intracerebral hematoma, which is associated with a poor outcome.[22–24]

Due to the complexity of the molecular processes that are involved in the onset of stroke, but also in ischemic reperfusion injury, multiple strategies are considered for treating stroke. These strategies include both acute and long-term approaches. Acute therapies aim to salvage the ischemic penumbra and limit reperfusion injury, while long-term therapeutic strategies aim to reconstitute the lost tissue from the ischemic core, as will be discussed later.

B. Limitations and Potential Improvements of Available Therapies for Ischemic Stroke

Current therapies or approaches that have been proven to be effective in reducing the mortality rate and improving the functional outcome of acute ischemic stroke include the establishment of a specialized stroke care unit (SCU),[25] thrombolysis with tissue plasminogen activator (tPA),[26,27] aspirin administration,[28] and decompressive surgery following ischemic stroke.[29] The most remarkable advance in stroke management that reduced the mortality and disability rate has been the establishment of a SCU, which is a separate physical space in general medical wards with specialized and specifically dedicated trained staff.[25] Despite these advances in stroke management, they can only be applied in a short therapeutic window. The FDA-approved standard treatment for stroke, thrombolysis with tPA, is only applicable to less than 10% of stroke patients and ideally needs to be initiated within 3 hr after the onset of ischemia. This low applicability rate of tPA is mainly associated with the higher risk of intracerebral hemorrhage when tPA is administered longer than 3 hr after the onset of stroke symptoms.[30] Moreover, patients often do not recognize stroke-associated symptoms and only show up at the hospital with advanced stroke symptoms[31] or the symptoms are not immediately recognized by the hospital staff that can be counteracted by a SCU.[25] Therefore, the presenting stroke patient has often exceeded the safe 3-hr time window to be considered for tPA treatment. This time window can be extended to 4.5 hr if low-risk patients are selected together with providing extensive care due to the higher risk of secondary damage and increased mortality due to reperfusion injury.[32,33] The major criteria to consider these low-risk patients included the absence of cerebral hemorrhage or major infarction and they presented with acute ischemic stroke and the symptoms started 3–4.5 hr before initiation of drug administration (for all inclusion and exclusion criteria, see Table I in Ref. [34]).

Other approaches such as decompressive surgery and aspirin administration need to be started within 48 hr after the onset of stroke. Moreover, the benefits of aspirin administration are small while decompressive surgery is only applicable for patients whose stroke-associated infarct region is caused by middle cerebral artery-related pathology, combined with malignant space-occupying brain edema.[35]

Various interventions are currently under investigation, which include extending the time window for thrombolysis with desmoteplase or alteplase,[36,37] ultrasound-enhanced thrombolysis,[38] the creation of new thrombectomy devices,[39] stents, stent retrievers, and protective drugs.[17] Asadi et al. provide an up to date, in depth overview of the different studies using endovascular treatments, which can be used as an important supplementary therapy to current intravenous thrombolysis.[40]

However, when extending the therapeutic window for thrombolytic interventions, the ischemic brain still needs to be protected from reperfusion injury. Therefore, reducing reperfusion injury is also a route that is currently being investigated as an additional procedure to be implemented after thrombolytic therapy.[20] These approaches aim at reducing the local production of ROS and BBB-damaging MMPs, or mediating the local immune response that would otherwise lead to secondary damage.[41–44] One of these approaches is therapeutic hypothermia[45] that has been shown to decrease ischemic and reperfusion injury by influencing local excitotoxicity, neuroinflammation, and ROS production.[46]

While these therapies and novel interventions aim at mitigating the disease outcome, they can only be applied in the first few hours or days after the onset of ischemic stroke.[32,47] Patients surviving stroke and not treated properly within this narrow time window are therefore often left with permanent disabilities, associated with the focal areas of the brain that are affected.[32] In these patients, a therapy that can be applied weeks to months after stroke onset can be beneficial. These therapies aim at restoring the lost neural tissue or stimulating brain plasticity to improve the functional outcome but also muscle strengthening and physical conditioning has been shown to improve the quality of life of patients with permanent disabilities.[48] Stem cell based therapies have been shown to be a promising approach in achieving such results.[49]

C. Stem Cell Sources and Mechanisms of Action for Cell-Based Therapies

When considering stem cells sources for a cell-based therapy in ischemic stroke, ex vivo expanded and manipulated neural stem cells (NSCs) or neural precursor cells (NPCs) would be

Table I. Overview of Preclinical Stroke Studies Using MSCs

Stem cell type	(Pre)differentiation and/or treatment	Species	Occlusion time	Time of transplantation	Cell dose and location of transplantation	Fate of transplanted cells	Outcome	Reference
h-DPSCs	No	Sprague–Dawley rats	2 hr	24 hr post-surgery	3×10^5 intrastriatal; 3×10^5 intracortical	2.3% migrates toward the stroke lesion. Differentiation toward astrocytes in preference to neurons	Improvement in forelimb sensorimotor function at 4 weeks posttreatment, mediated by paracrine effects	59
h-UMSCs	hUMSCs and hUMSCs cultured in neuronal conditioned medium	Sprague–Dawley rats	90 min	24 hr post-surgery	2.5×10^5 intracortical in two sites	36 Days survival, not quantified	Significant improvements in motor function, greater metabolic activity of cortical neurons, and better revascularization in the infarct cortex due to paracrine effects	71
h-UMSCs	No	Wistar rats	2 hr	24 hr post-surgery	2×10^5 intracortical	5 Weeks survival, <3% expressing neural markers	hUMSC accelerate neurologic recovery after stroke by promoting angiogenesis	130
h-BMSCs	No	SHR rats	Permanent	1 week post-surgery	7.5×10^4 in three different cortical sites	6 Weeks after grafting, donor cells expressed astrocyte, oligodendroglial, and neuronal markers. Functional integration was unlikely	Improved functional outcome, mediated by paracrine factors that are produced by the surviving donor cells	70
r-BMSC and h-BMSCs	Notch-induced BMSCs	Sprague–Dawley rats	1 hr	4–6 weeks post-surgery	6×10^4 intrastriatal in three sites	r-BMSCs show higher survival (15% vs. 7%) and differentiation than h-BMSCs	Improvement in locomotor and neurological function. Reduced loss of striatal periinfarct cells.	137
h-ASCs	No	C57BL/6J mice	1.5 rh	1 week post-surgery	5×10^6 in the stroke lesion	Large percentage hASCs express MAP2. Low percentage of GFAP expression	Cognitive recovery and decrease in infarct size. Immunomodulation by decreasing the presence of Iba-1+ microglia and GFAP+ astrocytes	142
m-ASCs	No	C57BL/6J mice	Permanent	24 hr post-surgery	1.8×10^4 above the corpus callosum	Migration after 1 week, toward vessels, 5% survival after 4 weeks	Ischemia induces ASC-survival, migration toward the lesion and microvessels, differentiation into smooth muscle cells	138
r-BMSC	Hypoxic pretreatment (HP)	C57BL/6J mice	Permanent	24 hr post-surgery	1.0×10^6 intranasal	1.5 hr after administration, donor cells were observed in the ischemic cortex. No long-term follow up was performed	HP of BMSCs induced a higher expression of migration associated and significantly reduced infarct size and improved sensorimotor function compared to non-HP BMSCs	140
r-BMSCs	No	Wistar rats	90 min	1, 6, 24, or 48 hr post-surgery	1×10^6 into the carotid artery	q-dot nanocrystal marked BMSCs could be detected 7 days poststroke	Injecting BMSCs 24 hr after stroke had the most significant effect on graft survival/integration, infarct size reduction, and improvement of neurological function. SDF-1 and bFGF were upregulated	127
r-BMSCs	No	Wistar rats	2 hr	24 hr post-surgery	2×10^6 into the carotid artery	n/a	BMSCs facilitate axonal sprouting and remyelination in the cortical ischemic boundary zone and corpus callosum	150

Continued

Table I. Continued

Stem cell type	(Pre)differentiation and/or treatment	Species	Occlusion time	Time of transplantation	Cell dose and location of transplantation	Fate of transplanted cells	Outcome	Reference
r-BMSCs	No	Wistar rats	2 hr	30 min after reperfusion	1×10^6 into internal carotid artery	Magnetically labeled BMSCs could be detected with MRI	Magnetically labeled IA-delivered BMSCs could be detected with MRI and high cerebral engraftment rates are associated with impeded cerebral blood flow after injection	143
h-BMSCs	No	Wistar rats	90 min	24 hr post-surgery	1.1×10^6 or 0.5×10^6 into the external carotid artery	Transient localization of engrafted cells in the host brain	Localization of BMSCs in the brain but relocated to other organs 24 hr later. Increased radioactivity counts in the ipsilateral stroke hemisphere	144
h-BMSCs	No	Sprague–Dawley rats	75 min	24 hr, 4 days, and 7 days post-surgery	1×10^6 into the carotid artery	Low survival, no expression of neuronal markers. Migration toward the lesion and secretion of BDNF	Time dependent functional recovery and cell distribution around the lesion. Mechanisms of action: neuroprotection, angiogenesis and enhancing reactive astrocytes, downregulation of MMP9	128
Autologous r-ASCs	No	Sprague–Dawley rats	90 min	3 days post-surgery	2×10^6 into the carotid artery	1.5% of surviving cells expressed NeuN; 1% survival of transplanted cells	Improvement of neurological deficits, migration of donor cells to lesion; attenuation of astroglial activity, inhibition of apoptosis, and promotion of cellular proliferation	131
r-BMSCs	No	Aged Wistar rats	2 hr	24 hr post-surgery	2×10^6 into the carotid artery	Donor cells survive up to 1 year and preferentially differentiate toward astrocytes	The beneficial effects of cell transplantation persisted for at least 1 year. Donor cells survived, differentiated toward astrocytes or neurons or colocalized with microglia and endothelial cells. Reduction of axonal loss and glial scar thickness	147
h-ASCs and r-ASCs	No	Sprague–Dawley rats	Permanent	30 min post-surgery	2×10^6 h-ASCs or r-ASCs	No migration/implantation of donor cells was observed	Improved functional outcome; reduction in neuronal cell death. No reduction in lesion size. VEGF and synaptofysin was upregulated and GFAP was downregulated in the treated groups. No difference was observed between the h-ASC- and r-ASC-treated groups	151
h-BMSCs	Immortalized cells	Wistar rats	1 hr	24 hr post-surgery	3×10^6 into the jugular vein	After 7 days no donor cells were detected	IV- transplanted human MSCs induced functional improvement, reduced infarct volume, and neuroprotection by providing IGF-1 and inducing neurotrophin expression in host brain	135

Continued

Table I. Continued

Stem cell type	(Pre)differentiation and/or treatment	Species	Occlusion time	Time of transplantation	Cell dose and location of transplantation	Fate of transplanted cells	Outcome	Reference
h-BMSCs and h-BMSC-EVs	No	C57BL/6J mice	30 min	24 hr post-surgery. Repeated after 3 and 5 days for hBMSC-EVs	1×10^6 BMSCs or EVs from 2×10^6 BMSC in the femoral vein	n/a	Mice receiving EVs showed improved neurological function and long-term survival associated with improved angiogenesis and neurogenesis, which resembled BMSC responses.	129
r-BMSCs	No	Wistar rats	2 hr	30 min after reperfusion	1×10^6 into the femoral vein	Magnetically labeled IV-delivered BMSCs could not be detected	Magnetically labeled IV-delivered BMSCs could not be detected	143
h-BMSCs	BMSCs, PlGF gene transfected MSCs	Sprague–Dawley rats	Permanent	6 hr post-surgery	1×10^7 intranvenously	LacZ-expressing PlGF-hBMSCs were found primarily in the penumbra and express NeuN (±10%) and GFAP (<17.23%)	hBMSCs and PlGF-transfected BMSCs improved angiogenesis, reduced the lesion size, and elicited functional improvement; the effect was more pronounced in PlGF-transduced BMSCs	136
r-BMSCs	BMSCs, CXCR4 gene transfected BMSCs, and siRNA-CXCR4-transfected BMSCs	Sprague–Dawley rats	2 hr	24 hr post-surgery	2×10^6 into the femoral vein	Increase in CXCR4-BMSCs surrounding the infarct compared to nontransfected and siRNA-CXCR4 transfected BMSCs	CXCR4-transfected BMSCs increased the periinfarct capillary bed, reduced the infarct size, and improved the functional outcome compared to nontransfected and siRNA-CXCR4-transfected BMSCs	141
r-ASCs	No	Sprague–Dawley rats	3 hr	0, 12, and 24 hr after stroke onset	2×10^6 intravenously	Migration toward the lesion, questionable differentiation toward endothelial cells	Reduction of infarct region. Improvement in sensorimotor function, upregulation of CXCR4 and SDF-1. Decreased apoptosis in infarct region	132
r-BMCS and r-ASCs	No	Sprague–Dawley rats	60 min	30 min after reperfusion	2×10^6 into the femoral vein	Migration of transplanted cells toward the lesion was not observed	BMSC and ASC administration improves functional recovery independent of reducing the infarct volume and cell migration. Treated groups show higher cell proliferation, oligodendrogenesis, synaptogenesis, and angiogenesis markers	60
m-BMSCs and m-ASCs	No	C57BL/6J mice	90 min	Immediately after reperfusion	1×10^5 ASCs or BMSCs into the tail vein	n/a	ASC administration attenuated ischemic damage. Incomplete ASC incorporation in the brain. HGF and angiopoietin-1 expression was significantly increased in ASC-treated mice compared with the BMSC group	139
h-BMSCs	No	Sprague–Dawley rats	90 min	7 days post-surgery	$3.4 \pm 1.2 \times 10^6$ into the saphenous vein	Donor cells accumulate in the ischemic hemisphere, but also in the spleen and lungs	IV-injected 99mTc-HMPAO-labeled MSCs home to the ischemic lesion but also accumulate in the lungs and the spleen	145

Continued

Table I. Continued

Stem cell type	(Pre)differentiation and/or treatment	Species	Occlusion time	Time of transplantation	Cell dose and location of transplantation	Fate of transplanted cells	Outcome	Reference
h-BMSCs	No	Sprague–Dawley rats	Hours, no details	60 days post-surgery	4×10^6 in the jugular vein	Donor cells preferentially migrate to the spleen, up to 12 days postinjection	Significant reduction in striatal and periinfarct area. Reduced loss of hippocampal neurons, significant reduction in MHC-II activated inflammatory cells in gray and white matter. TNF-alfa expression in the spleen was decreased	134
r-BMSCs	No	Sprague Dawley rats	90 min	1, 4, or 7 days post-surgery	3×10^6 intravenously	Cells transplanted 1 day after stroke migrated toward the cortex, cells transplanted after 4 days or 7 days migrated to the striatum	Functional recovery (mNSS score) was highest when cells were transplanted 1 day after surgery. This was correlated with a time-dependent expression of SDF-1 and MCP-1 between ischemic regions	133
r-BMSCs	No	Aged Wistar rats	2 hr	1 month after surgery	3×10^6 intravenously	Preferential differentiation toward astrocytes (13%) over neurons (6%). Survival of donor cells was not quantified	Significant sensorimotor and general neurological recovery after cell compared with control animals. BMSC treatment reduced scar thickness, and increased the number of proliferating cells and oligodendrocyte precursors. SDF-1 is upregulated in the ischemic boundary zone after stroke. BMSCs express CXCR4	146
r-BMSCs from SHR-SP rats	No	Aged SHR-SP rats	Permanent	30 days before stroke onset	5×10^5 into the tail vein	No direct transplantation, injected donor cells prior to MCAO	SHR-SP BMSCs transplantation increased microvasculature density in the periinfarct zone, reduced ischemic brain damage, and improved neurologic function. Rejuvenation of bone marrow from aged rats with young cells enhanced the ischemic response at the level of endothelial/vascular activation	152
h-UTCs	No	Aged Wistar rats	Permanent	24 hr post-surgery	1×10^7 cells/kg into the tail vein	Very few donor cells present at lesion site, no reactivity for MAP2 or GFAP	IV administration of hUTC improved neurological functional recovery without reducing infarct size, increased progenitor cell proliferation and vessel density in the ischemic boundary zone, and enhanced synaptogenesis	148
r-BMSCs, h-BMSC	No	Aged Sprague–Dawley rats	3 hr	6 hr post-surgery	1×10^6 cells/kg into the tail vein	1% migrates toward the lesion	Daily treatment with G-CSF improved neurological function. G-CSF + BMSC transplantation stimulated angiogenesis in the infarct core but did not further improve neurological function or infarct volume size	149

IGF-1, insulin-like growth factor 1; HGF, hepatocyte growth factor; SHR, spontaneous hypertensive rats; SHR-SP, stroke-prone SHR; 99mTc-HMPAO, 99-technetium bound to hexamethylpropylene amine oxine; hUTCs, Human umbilical tissue derived cells; EV, extracellular vesicles; MCP-1, monocyte chemotactic protein-1; TNF-alfa, tumor necrosis factor alfa; prefix h, human; m, mouse; r, rat.

the ideal candidates to stimulate repair in the central nervous system (CNS) due to their neurogenic differentiation potential and predisposition.[50–53] Promising results have already been achieved with human NSCs in animal models of neurological disorders, including stroke.[8,54] However, there is a need for alternative stem cell sources with regenerative potential due to ethical considerations with regard to the isolation of NSCs from embryonic or fetal tissue together with isolation and culturing complications of adult NSCs.[55,56] These alternative stem cells need to be able to reconstitute the lost tissue or stimulate endogenous repair. The most promising alternatives for NSC that are of nonembryonic or nonfetal origin are mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSCs), and bone marrow mononuclear cells (BMMNCs). These stem cell sources have shown to possess regenerative effects on the brain and allogeneic transplantation potential[57–63] Moreover, these stem cell types can be obtained by means of minimal invasive procedures thereby reducing donor site morbidity during isolation. Although it remains a topic of debate whether MSCs possess NPC properties, several studies have reported the ability of subtypes of MSCs to acquire neuronal features following exposure to the proper environmental stimuli.[64–66] In addition to the discussion of which stem cell source is most suitable for stroke research, different animal models such as the middle cerebral artery occlusion (MCAO) model or photothrombotic stroke model are available to induce stroke in an experimental setting, each with their own strengths and weaknesses.[67–69]

Multiple mechanisms have been proposed for stem cell mediated therapies, including brain protection, cell replacement, immunomodulation, and promoting both brain plasticity and angiogenesis in damaged brain regions (Fig. 2).[49] Interestingly, these mechanisms are mainly thought to be mediated by the effect of the stem cell secretome on endogenous stem cells and on the host microenvironment instead of directly replacing the lost cells,[59,70,71] although encouraging results have also been achieved with cell replacement studies.[57,58] Therefore, the transplanted cells can be seen as a vehicle for sustained growth factor delivery at the stroke lesion, which can also respond dynamically to changes in the local microenvironment as will be discussed next and into more detail in the following sections.

Stem cell mediated neuroprotective effects have been observed in in vitro and in vivo models of neurological disorders.[72–74] These neuroprotective effects are mainly attributed to the soluble factors secreted by the stem cells. In addition to the development of protective therapies, interventions aiming at the directed recruitment and differentiation of NSCs to the site of injury are considered. It is known that NSCs are present in the subventricular zone and dentate gyrus of the hippocampus in the adult brain.[53,75,76] Moreover, following ischemic stroke, endogenous NSCs differentiate into neurons and migrate toward the site of stroke injury and contribute to brain repair.[77,78] A determining factor in the directed migration of neurons is stromal cell derived factor α1 (SDF-1) and its receptor CXCR4.[78,79] This SDF-1/CXCR-4 axis has been shown to act as an inflammatory mediator after acute cerebral ischemia[80,81] but has also been shown to play an important role in CNS development,[82] has a modulating effect on different subsets of neurons,[82] and has strong effects on cell migration, axon guidance, and angiogenesis in the postacute phase of stroke.[79,82]

Unfortunately, the endogenous repair by NSCs is insufficient to completely replace the lost tissue. Therefore, in addition to exerting a protective effect on the brain, novel cell based therapies are focusing on improving the recruitment of and repair by endogenous NSCs and supporting cells.[8] Direct cell replacement by neurons derived from stem cells themselves is also a route that is being considered, although it is uncertain whether the transplanted stem cells are able to survive and adequately integrate into the host brain.[57,59] It has been suggested that damaged areas in the brain can only be successfully reconstituted by the equivalent homotopic neurons, which stresses that adequate pretransplantation targeted differentiation of stem cells grafts toward specific types of neurons is required for direct cell replacement by the stem cells themselves.[83–85] For example, it has been shown that grafting cortical donor tissue into the

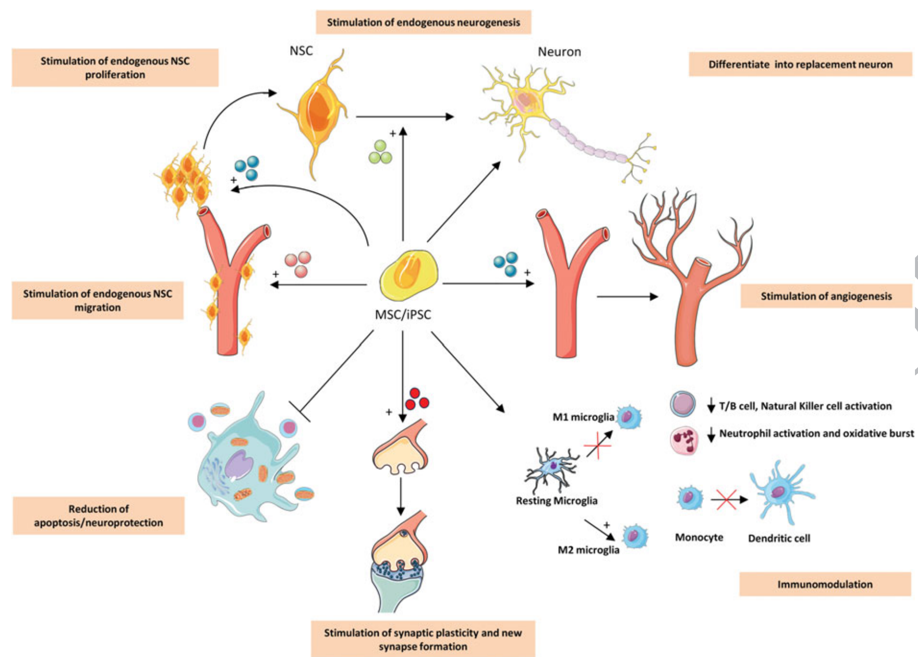

Figure 2. Mechanisms of action of cell-based therapies in ischemic stroke. The poststroke microenvironment can be modulated by exogenously delivered stem cells by multiple mechanisms to trigger tissue repair. Stem cells can contribute to poststroke recovery by stimulating the migration of endogenous NSCs toward the stroke lesion, where proliferation and differentiation toward replacement neurons can be triggered. In addition, transplanted stem cells are thought to be able to replace the lost neurons themselves in addition to stimulating host NSCs. Moreover, the formation and attraction of new blood vessels toward the ischemic lesion and the stimulation of synaptogenesis and synaptoplasticity contributes to host repair. In addition to directly stimulating the formation of new brain tissue, the degradation of existing cells in, for example, the ischemic penumbra is inhibited by neuroprotective mechanisms such as ROS scavenging by the transplanted cells. Immunomodulatory effects are also observed and include the inhibition of neutrophil activation and migration, effector T-cell and B-cell inhibition, reducing the activation and attraction of peripheral dendritic cells, and stimulating the M2 microglial phenotype. These effects are predominantly caused by the soluble factors released by the stem cells, but also cell–cell interactions appear to play a role. Image was created using Servier Medical Art.

damaged motor cortex reestablished cortical and even subcortical circuitry,[84] a feature that was not observed when using heterotopical tissue such as occipital cortex.[86] More recently, a study by Michelsen et al. demonstrated that in vitro differentiated mouse embryonal stem cell (ESC) derived visual cortical neurons were able to reestablish connections with the damaged visual cortex with recriprocal axonal projections and synaptic integration.[83] Interestingly, grafting these cells in the damaged motor cortex or ESC-derived motor neurons in the damaged visual cortex did not lead to graft integration.[83] In addition, targeted differentiatiated iPSCs to pyramidal cortical neurons have been shown to integrate in the host circuitry after transplantation into to the neonatal mouse brain[87] and have been used in preclinical stroke research,[57] as will be discussed later. Similarly, it has been suggested that potential donor cells for Parkinson's disease should be of the correct nigral dopaminergic neuron phenotype to improve functional engraftment with the appropriate targets.[85] Another theory in which cell-based therapies are believed to improve the functional outcome in stroke is by directly inducing brain plasticity after the ischemic insult. Although studies report the functionality of transplanted cells in the endogenous neuronal circuitry, these effects are thought to be mainly mediated by promoting the formation of new synapses between existing neuronal cells and not by functionally integrating into the host neuronal network.[57,58,87,88]

A key concept in the regeneration of lost tissue is establishing adequate blood supply to the regenerating tissue. Without proper vascularization that provides oxygen and nutrients, the newly formed neuronal tissue will be unable to survive. Previously, it has been shown that stem cells can form vascular structures in vitro and secrete proangiogenic factors that can positively influence the growth of blood vessels in vitro and in vivo.[89–91] Therefore, stimulating angiogenesis is another mechanism by which cell-based therapies can influence stroke outcome. Remarkably, revascularization appears to be the main mechanism by which BMMNCs are able to ameliorate the disease outcome.[61–63]

In addition to protecting damaged neurons, restoring the lost neuronal circuitry and blood supply, stem cells have been shown to be able to mediate the immune response.[92,93] The mechanisms of these immunomodulating properties include influencing the activation state of monocytes, natural killer cells, B cells, T cells, and neutrophils. Stem cells were also shown to mediate immunoglobulin release from plasma cells and upregulate the amount of regulatory T cells.[92–94] However, it is important to take into account that in ischemic stroke, one of the most common causes of stroke-related morbidity is severe systemic immunosuppression, making patients susceptible to infections.[41] Therefore, additional systemic immunosuppression by cell-based therapies could worsen stroke outcome. Fortunately, no adverse effects on systemic cytokine levels were observed following stem cell transplantation in a rat model of stroke.[94]

Despite the promising results with BMMNCs in ischemic stroke from preclinical studies,[61–63,95,96] in vitro evidence of the effect of BMMNCs on the above-mentioned mechanisms is scarce.[97,98] Therefore, this review will focus on MSCs and iPSCs as readily available sources of stem cells and compare their efficacy and potential to ameliorate the disease outcome in animal models of ischemic stroke. In addition, novel imaging strategies allowing in vivo tracking of transplanted cells and noninvasive evaluation of brain repair following stroke will be discussed.

2. MESENCHYMAL STEM CELLS AS A THERAPY IN STROKE

MSCs, initially discovered in the bone marrow stromal cells (BMSCs) by Friedenstein et al. in the late 1960s,[99] were later found to be able to differentiate toward cells producing mes- Q4
enchymal tissues including bone-forming osteoblasts, cartilage-producing chondroblasts, and adipocytes.[100] In addition to bone marrow, MSCs have been isolated from a varying range of other tissues including but not limited to adipose tissue (ASCs), Wharton's Jelly in the umbilical Q5
cord (UMSCs), umbilical cord blood, and dental tissues.[101–105] Additional research into the differentiation capacity of MSCs suggested that these cells were able to differentiate toward hepatocytes,[106] cardiomyocytes,[107] and neuron-like cells.[108] The presence of MSCs in various easily accessible and available donor tissues such as the dental pulp and adipose tissue makes MSCs a promising cell type for stem cell based therapies. However, the main problem in the extensive research with MSCs is the difficulty to compare study outcomes between different research groups. Research groups often have their own methods of isolating, expanding, and characterizing the cells, leading to diverging criteria to define MSCs.[101–103,109,110] MSCs are a heterogeneous population that generally express the surface markers, CD29, CD44, CD90, CD117, and CD146, while they do not express CD34 and CD45 although subpopulations of CD45- and CD34-expressing MSCs were identified.[111]

A. In vitro Evidence for the Regenerative and (Neuro)protective Potential of MSCS on the Brain

Despite interlaboratory differences in defining and culturing MSCs, researchers agreed on the multilineage differentiation potential[100,101,112] of these stem cells and subsequently investigated the ability of these cells to transdifferentiate into neuronal or neural-like cells in order to obtain a cell source to replace the lost tissue after ischemic stroke. Early studies that investigated the neurogenic differentiation potential of MSCs were performed using BMSCs, but also hASCs and dental tissue and umbilical cord derived stem cells were successfully differentiated to cells with a neuronal phenotype, expressing markers such as neuronal nuclei (NeuN), microtubule-associated protein 2 (MAP2), neural cell adhesion molecule, and synapsin I.[66,108,113,114] Although a consensus was not found between the differentiation protocols, epidermal growth factor and basic fibroblast growth factor (bFGF) are thought to play an important role in inducing MSCs toward a neuronal cell lineage.[66,113] Subsequent maturation of the induced cells was based on increasing intracellular cyclic adenosine monophosphate and protein kinase C signaling or by specific growth factor administration.[66,108,113–115] However, few studies performed electrophysiological measurements on the differentiated cells but were able to report both voltage-gated sodium and potassium currents that could be reversibly blocked by tetrodotoxin and tetraethylammonium, respectively.[66,108,113,115] Of these studies, only the studies by Wislet-Gendebien et al.[108] and Gervois et al.[66] could demonstrate the ability of the differentiated cells to generate a single action potential in neuronally differentiated BMSCs and human dental pulp stem cells, respectively, demonstrating only incomplete neuronal differentiation.

In addition to the neuronal differentiation capacity of MSCs, researchers also investigated the neuroprotective and regenerative potential of the MSC secretome. Hypoxia- and glutamate-induced excitotoxicity assays were used as in vitro models for ischemic stroke. It was shown that the MSC protect SH-SY5Y neuroblastoma cells against hypoxia- and glutamate-induced excitotoxicity both in coculture assays and assays using conditioned medium of MSCs, suggesting paracrine effects.[72,74,116,117] Although the influence of the MSC secretome on NSC survival and/or differentiation is not evaluated in vitro, the MSC secretome has been shown to stimulate neurite outgrowth in dorsal root ganglia (DRG)[118,119] and axotomized retinal ganglion cells (RGCs),[73] and to enhance survival of these axotomized RGCs[73] and primary cortical[120] and dopaminergic neurons.[121] Neurotrophins/growth factors that are secreted by MSCs include glial-derived neurotrophic facto, neurotrophin-3 (NT-3), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF).[66,73,118–122] These factors are believed to play an important role in neurite outgrowth of DRGs[118,119,122] and axotomized RGCs.[73] Moreover, these factors are also suggested to protect RGCs from neurodegeneration after axotomization,[73] cortical neurons from nitrix oxide exposure and withdrawal of trophic support,[120] and dopaminergic neurons from 6-hydroxy-dopamine.[121]

As mentioned previously, another key concept in promoting brain regeneration after ischemic stroke is stimulating revascularization of the regenerating tissue. Therefore, the influence of the MSCs was not only investigated in (neuro)protective or neurite outgrowth assays but also the ability of the MSCs to stimulate angiogenesis was evaluated. These studies showed that MSCs are able to stimulate tube formation and endothelial cell migration, enhance wound healing, and improve blood vessel formation in the chorioallantoic membrane essay.[89,123,124] These proangiogenic properties of MSCs were attributed to the soluble factors that are secreted by the cells (Table I in Refs. [89] and [90]) Furthermore, it was shown that MSCs protect endothelial cells against hypoxia-induced cell death.[125] Several studies also suggest that MSCs not only promote angiogenesis by paracrine effects but that these cells are also able to differentiate into endothelial cells (Table II in Ref. [89]).

Although neurogenic differentiated MSCs express neuronal markers, differentiation of these cells toward mature neurons appears limited, as only immature electrophysiological profiles can be generated from these cells. Nonetheless, MSCs show great promise in vitro as shown by the neuroprotective and proangiogenic effects of the MSC secretome. Therefore, several studies have transplanted MSCs into animal models of ischemic stroke and evaluated the outcome, as will be discussed below.

Table II. Preclinical Studies with Neurogenic Predifferentiated iPSCs

Stem cell type	(Pre)differentiation and/or treatment	Species	Occlusion time	Time of transplantation	Cell dose and location of transplantation	Fate of transplanted cells	Outcome	Reference
h-iPSCs	iPSC-derived lt-NES cells	Nude Rats	30 min	48 hr post-surgery	2×10^5 instrastriatal or 1.5×10^5 intracortical in two sites	>50% of grafted cells survive up to 4 months posttransplantation. Intrastriatal injected cells differentiate toward neurons in preference to astrocytes (72.9% vs. 1.9%) 4 months posttransplantation). Of the intracortical injected cells, 78.5% of cells in the graft core were NeuN positive while 13.4% were DCX-reactive in the periphery	Cells grafted into the striatum survive up to 4 months, differentiate to functional neurons, and receive synaptic input from host cells. Intracortical transplanted cells survive for 4 months and differentiate into functional neurons that receive synaptic input from host cells in T-cell-deficient rats without forming tumors	58
h-iPSCs	iPSC-derived lt-NES cells	C57BL/6 Mice	30 min	1 Week post-surgery	1×10^5 intrastriatal	Approximately 10% survival after 10 weeks; 78.5% HuD-positive cells found at graft core, 13.4% DCX-positive cells in the graft periphery	Improvement of motor function, independent of long-term graft survival mediated by enhancing endothelial plasticity. Remaining grafted cells differentiate toward neurons and integrate into the host brain	58
h-iPSCs	iPSC-derived lt-NESCs fated into cortical neurons and nondifferentiated iPSC-derived lt-NESCs	Sprague–Dawley and nude rats	30 min	48 hr post-surgery	1.5×10^5 intracortical in two sites	Transplanted cells survive 2 months posttransplantation. Fated cells are fewer and proliferate less than nonfated cells but express a higher percentage of the mature neuronal marker NeuN (13.7% vs. 7.1%) and the cortical marker TBR1 (2.5% vs. 1.4%)	iPSCs-derived cortical neurons survive, differentiate to functional neurons, and improve neurological outcome after intracortical implantation without tumor formation in a rat stroke model	57

Continued

Table II. Continued

Stem cell type	(Pre)differentiation and/or treatment	Species	Occlusion time	Time of transplantation	Cell dose and location of transplantation	Fate of transplanted cells	Outcome	Reference
h-iPSCs	iPSC-derived lt-NES cells	Aged Sprague–Dawley rats	30 min	48 hr post-surgery	1.5×10^5 intracortical in two sites	49.2 % of the grafted cells survive 8 weeks posttransplantation, 30% of transplanted cells express DCX in the periphery, 91.3% express HuD, and 19.6% express GABA outside the graft core	Cell-grafted showed increased sensorimotor function compared to vehicle-treated rats. Transplanted lt-NES cells expressed markers of neuroblasts, mature and GABAergic neurons. Microglia activation was diminished in lt-NES grafted rats. Neuronal loss was diminished after transplantation	170
h-iPSCs	iPSC-derived NPCs	Sprague–Dawley rats	90 min	1 Week post-surgery	1×10^5 intrastriatal	Survival not quantified, grafted cells express Sox2, nestin, Pax6, and extend MAP-2 expressing processes into the perilesional parenchym	Grafted iPSC-NPCs initially exert trophic effects on host brain structures, followed by iPSC-NPCs integration into the host brain	169
h-iPSCs	iPSC-derived NSCs	Sprague–Dawley rats	2 hr	Immediately after reperfusion	1×10^6 intrastriatal	Transplanted cells survived and migrated into the damaged host tissue and express nestin (51.4%) and beta III tubulin (44.3%)	Engrafted cells survive, migrate, and differentiate toward neuronal cells. Transplanted cells improved behavioral and sensorimotor function	164
h-iPSCs	iPSC-derived NSCs	C57BL/6J mice	1 hr	24 hr post-surgery	1×10^5 intrahippocampal	Engrafted cells migrated toward the site of injury. Survival % not quantified	Improved motor and sensorimotor function in graft-receiving animals. Mechanisms of action include a decrease in proinflammatory markers, adhesion molecules, and microglial activation BBB damage was attenuated	172
h-iPSCs	iPSC-derived NPCs	Wistar rats	30 min	1 Week post-surgery	2.5×10^5 intracerebral	Double amount of cells in the graft of which 41% were beta III tubulin/MAP2 positive; 5% of the grafted cells expressed GFAP	No migration of cells toward the lesion. No significant difference in behavioral recovery. Tumor formation was not present	171
h-iPSCs	Unclear	C57BL/6N and NSG-mice	Phototrombotic stroke	1 Week post-surgery	1×10^5 iPSC in the stroke cavity with or without hyaluronic acid hydrogel	38% versus 30% survival of cells with or without hydrogel 1 week after transplantation in NSG-mice. Grafted cells form DCX-positive neuroblasts	No assessment of functional recovery. Tumor formation was not evaluated	173

Lt-NES, long-term expandable neuroepithelial-like stem cells; NSG-mice, NOD scid gamma immunodeficient mice; prefix h, human; m = mouse.

med21400 W3G-med July 9, 2016 9:43

B. MSCS as a Therapy for Stroke In vivo

Due to the encouraging in vitro results of MSCs in protecting damaged neurons and stimulating revascularization in addition to secreting multiple soluble factors, the potential of different subtypes of MSCs to ameliorate stroke outcome after transplantation was evaluated in vivo (Table I). These studies evaluated the functional outcome after transplantation with a variety of behavioral tests. These tests include global neurological assessments to evaluate the disease severity such as the Bederson test and the modified neurological severity score (mNSS). In addition, specific motor, sensorimotor, and cognitive tests were performed. For detailed information on behavioral and disease severity tests in animal models of stroke, see Schaar et al.[126] Taken together, the studies that report an improvement in general neurological, sensorimotor, motor, or cognitive function are described in Table I.

The proposed underlying mechanisms responsible for the improvement in stroke outcome suggested by the studies listed in Table I are diverse. One of the possibilities was that the transplanted cells migrated toward the stroke lesion and differentiated locally toward neurons, establishing new connections with the host environment, [127] although the majority of the in vivo studies using MSCs as a therapy for stroke support paracrine mediated brain regeneration.[59,128,129] Regardless of the administration route, the fate of the transplanted cells was tracked using markers such as DiI,[60,130–133] DiR,[134] q-dot[127] or bromodeoxyuridine (BrdU)[135] incorporation prior to transplantation; LacZ[136] or GFP transduction;[59,70,137–142] iron particle[60,143] or radionuclide labeling;[144,145] in situ hybridization with the Y chromosome when male donors were used in a female host[146,147]; or antibodies directed against human mitochondria or human nuclei when human MSCs were grafted in a rodent stroke model.[71,128,148,149] While the listed studies could observe improvement of stroke outcome after transplantation, the amount of engrafted cells that was present in the stroke lesion was limited. Pioneered by Zhao et al., intracranial transplantation of MSCs showed that MSCs migrated toward the brain infarct region and were able to survive in the host brain and promote functional recovery.[70] Additional studies showed that although they were present only in low numbers,[59,71,130,137,138] the transplanted cells locally differentiated toward neural cells with a predisposition toward astroglial cells in preference to neurons.[59,130,137] Despite the local delivery of the transplanted cells in the stroke lesion, functional integration and replacement of the lost neural circuitry does not appear to be the mechanism of action of intracerebral transplanted MSCs to improve stroke outcome. The results of these studies suggest that the soluble factors secreted by the MSCs are the main actors in improving the functional outcome.[59,70,130,137] After cerebral transplantation of the MSCs, improved angiogenesis,[71,130,138] increased neuronal activity,[71] reduced loss of periinfarct cells,[137] and immunomodulatory effects[142] were observed. After transplantation, the local levels of soluble factors such as BDNF, vascular endothelial growth factor (VEGF), bFGF, and angiopoetin-2 were elevated.[71,130] In order to increase the regenerative potential of intracerebral administered MSCs, several alternative research approaches were investigated. For example, umbilical cord matrix stem cells were cultured in the presence of conditioned medium obtained from a 5-day culture of rat-derived neural cells. However, this approach did not lead to an additional improvement of stroke outcome.[71] Alternatively, it was shown that hypoxic preconditioning of MSCs promotes the survival, migration, and homing of the transplanted cells toward the ischemic lesion compared to nonpreconditioned cells.[140] Transplantation of these cells also leads to an additional functional improvement after intranasal administration, which is assumed to be mediated by an increase in the expression of migration-related proteins such as CXCR4 and MMPs in the hypoxic preconditioned stem cells.[140]

Intracerebral administration of MSCs is an invasive procedure and can lead to iatrogenic damage. Therefore, systemic administration of the transplanted cells via the arterial or venous route was considered. Intraarterial administration (IA) of MSCs in stroke has shown beneficial effects in animal stroke models. Interestingly, IA-transplanted cells are able to cross the BBB after ischemic stroke and migrate toward the stroke lesion. Even though MSCs were found in the core and periinfarct zone and expressed both astrocyte and neuronal markers,[128,131,147] paracrine mechanisms of action of IA-delivered MSCs are thought to be responsible for the enhanced stroke outcome, although integration into the host brain was observed but not functionally confirmed.[127] At the ischemic boundary zone, MSCs are only present in low numbers but were found to enhance axonal sprouting and remyelination,[150] angiogenesis, and BDNF production while decreasing MMP-9 levels and suppressing microglial activity.[128] Furthermore, BMSC administration into aged rats showed a sustained effect and donor cell survival up to 1 year after transplantation.[147]

Another route of MSC transplantation is via the venous system. Remarkably, MSCs that were intravenously (IV) delivered, improved the functional outcome after stroke without MSCs being observed in the ischemic brain or in lower numbers than in IA or intracerebral transplantation.[60,135,139,148,149,151] Despite being almost absent in the ischemic brain, IV transplantation of MSC induced an increase in periinfarct zone microvasculature density and the expression of proangiogenic factors[60,135,139,148] and improved oligodendrogenesis and synaptogenesis.[60,148,152] Remarkably, when BMSCs were transplanted IV in a chronic stroke model 60 days after surgery, the beneficial effects of the graft were attributed to their immunomodulatory effects on the stroke lesion and on the spleen where the cells preferentially homed toward.[134] Another successfully applied therapeutic approach was to transduce BMSCs with an adenoviral vector for PlGF[136] or a lentiviral vector for CXCR4,[141] which further improved the functional outcome after stroke compared to nontransduced BMSCs. The SDF-1/CXCR4 interaction in chemotaxis was found to be stimulated after intravenous administration of MSCs and plays an important role in MSC-homing toward the stroke lesion.[133,141,146] In accordance with the other routes of administration, the paracrine effects of the transplanted cells appear to be responsible for the posttransplantation effects on stroke outcome. This was supported by a recent study by Doeppner et al., who showed that repeated IV administration of extracellular vesicles produced by BMSC led to a similar improvement as injecting the stem cells themselves.[129] Moreover, this study also showed reduced poststroke immunosuppression after EV transplantation.

This overview shows a variety of studies that were performed using subtypes of MSCs and different administration routes. Although similar results were achieved with different subtypes of MSC, it remains debatable which is the most suitable source for MSC. Moreover, MSCs showed great promise in replacing the neuronal tissue after transplantation due to their in vitro neurogenic differentiation potential. However, cell replacement could not be proven in vivo and it is assumed that paracrine factors are responsible for the beneficial effects after MSC injection, which mainly stimulate angiogenesis and protect the host environment against additional damage without adequately stimulating endogenous neurogenesis or replacing lost neurons. Therefore, additional stem cell sources are considered that are able to differentiate to functional mature neurons in vitro and can potentially improve stroke outcome more effectively than MSCs.

3. INDUCED PLURIPOTENT STEM CELLS AS A THERAPY IN STROKE

In 2006, Takahashi and Yamanaka successfully transformed murine and in 2007, human fibroblasts to pluripotent stem cells by retroviral transduction with the transcription factors Oct3/4, Sox2, Klf4, and c-Myc, the so-called Yamanaka factors, allowing the formation of a patient-specific source of pluripotent stem cells.[153,154] These iPSCs are able to differentiate toward tissues from all three germ layers in vitro and in vivo as proven by teratoma formation upon subcutaneous transplantation of iPSCs.

The ability of iPSCs to differentiate into tissues of all three germ layers offers numerous potential therapeutic approaches. However, the main drawback in using iPSCs for transplantational studies in stroke research is that these cells, such as human ESCs, form teratomas when injected in an undifferentiated pluripotent state, with little to no improvement of the disease outcome.[155,156] Therefore, iPSCs are irreversibly predifferentiated in vitro in order to minimize tumor formation and improve the functional outcome as only the undifferentiated iPSCs form teratomas. However, a recent study by Choi et al. showed that iPSC- derived NPCs reactivate the silenced exogenous retroviral genes caused by a downregulation of DNA methyltransferases during differentiation and can return toward their pluripotent and thus tumorigenic state.[157] Moreover, nondifferentiated iPSCs can remain present within an iPSC-derived progenitor cell pool, which showed teratoma formation after subcutaneous transplantation. This teratoma formation was not observed when fully committed iPSC-derived cells were transplanted.[156] This study by Liu et al. was supported by Fu et al., who demonstrated that residual nondifferentiated iPSCs could not be eliminated by extended cell differentiation.[158] Therefore, adequate full neuronal commitment monitoring prior to transplantation is advised and studies that used nondifferentiated iPSCs in stroke research will be left out of this overview.

A. In vitro Evidence for the Regenerative and (Neuro)protective Potential of IPSCS on the Brain

The multilineage differentiation potential of iPSCs makes these cells an attractive alternative for NSCs as a source for cell-based therapies in neurological disorders. Neurogenic differentiating iPSCs follow similar developmental principles as hESC-derived neurons, although the neural differentiation efficiency can differ between different iPSC lines.[159] Targeted differentiation of iPSCs toward neuronal subtypes has been achieved by various research groups, using different approaches to generate a variety of neuronal cells including medium spiny neurons,[160] dopaminergic neurons,[161] motor neurons,[159,162] nociceptors,[163] and pyramidal cortical neurons.[57,87] Furthermore, it was shown that these neuronally differentiated cells are capable of repeated action potential firing, suggesting advanced maturation.[57,87,159,161,163] More importantly, this targeted differentiation of iPSCs toward specific neurons is of high importance and provides additional regenerative potential as it has been suggested that damaged brain areas can only be successfully repaired by the neurons corresponding to the damaged area, as discussed earlier.[83–85] Similar to MSCs, various differentiation protocols were developed to induce neurogenic differentiation of iPSCs. The protocols that were most successful are based on retinoic acid and sonic hedgehog signaling or based on SMAD inhibition using Noggin, which blocks SMAD signaling by the transforming growth factor beta superfamily of signaling proteins.[87,159,163,164]

In contrast to MSCs, where the paracrine effects of the stem cell secretome have been investigated thoroughly, there is a lack of stroke-related in vitro evidence of the (neuro)protective, regenerative, and angiogenic properties of iPSCs. Among the few studies that investigated the secretome of iPSCs, it was shown that iPSCs locally enhanced the production of proangiogenic factors. However, these studies did not include an in vitro secretome analysis or an in vitro evaluation of the angiogenic properties of the iPSC secretome.[165,166] Although the paracrine effects of the iPSC secretome on angiogenesis were not evaluated in vitro, several studies have reported endothelial cell differentiation of iPSCs,[91,167,168] which opened up the possibility that transplanted iPSCs can directly contribute to establish new blood vessel formation in the damaged brain.

Despite the lack of in vitro evidence for paracrine-mediated regeneration, the successful differentiation of iPSCs toward endothelial cells, neuronal precursors, and mature neuronal subtypes encouraged the use of iPSCs in animal models of ischemic stroke.

B. Neurogenic Predifferentiated IPSCS in In vivo Stroke Models

Contrary to MSCs, iPSCs that were transplanted in animal stroke models were neuronally differentiated prior to engraftment in order to avoid teratogenicity (Table II). Moreover, no reports are available in which iPSCs are administered IV or IA. Information about mechanisms of action of iPSCs in ameliorating stroke outcome is therefore only available for intracranially transplanted neurogenic predifferentiated iPSCs.

When considering the overall results of the behavioral tests performed after iPSC transplantation after stroke, improvements in general neurological score,[169] motor,[57,58,164,169,170] sensorimotor,[57,58,164,169,170] and cognitive function[164] were observed. On the contrary, Jensen et al. did not observe an iPSC-mediated improvement in behavioral function.[171]

Underlying mechanisms of action of the transplanted iPSCs include migration and local neuronal maturation of the transplanted iPSCs,[57,58,164,170] synaptic integration into the host brain[57,58] but also paracrine effects are considered to play a role.[169] The transplanted iPSCs were predifferentiated toward long-term expandable neuroepithelial-like stem cells,[57,58,170] fated toward cortical neurons[57] or toward neuronal precursor cells.[164,169,171] Transplanted iPSCs were traced with antibodies directed against human nuclei, cytoplasm or mitochondria,[57,58,169–172] DiI labeling,[164] or by using GFP positive iPSCs.[57,58,173] The engrafted cells survived up to 10 weeks after transplantation but the percentage of surviving cells varied considerably between the different studies, which can be caused by several factors. For example, the host strain (i.e., nude rats vs. immunocompetent rats) and species[174–177] can have an influence on the stroke outcome and cell survival rates.[58] Moreover, the altered stroke microenvironment in aged animals might be less favorable for cell transplantation.[170,178] Another factor that can influence cell survival and amelioration is the time after stroke onset; the transplantation is performed as cell transplantation at later time points exposes the cells to the established immune response,[179] whereas cell transplantation early after stroke onset or too close to the ischemic core can expose the cells to limited blood supply, oxidative stress, and trophic factor deficiency.[13] Remarkably, both engrafted fated cortical neurons derived from long-term expandable neuroepithelial-like stem cells and their nonfated counterparts, which were transplanted 48 hr after stroke onset, could be detected 2 months posttransplantation in the damaged rat cortex in the study by Tornero et al.[57] The transplanted cells differentiated locally toward neuronal cells as shown by the expression of doublecortin (DCX) at the periphery of the graft. The expression of HuD and NeuN was found at the core of the graft,[57,58,170] which was increased by differentiating the long-term expandable neuroepithelial-like stem cells toward cortical neurons prior to transplantation.[57] A study by Lam et al. demonstrated that 1-week survival of the iPSC grafts is enhanced—although not significant—in photothrombotic stroke by delivering the cells in a hyaluronic acid hydrogel. However, despite claiming to use iPSC-derived NPCs, the neuronal characteristics were not described, which might have had an impact on the survival rate. Additionally, transplantation of the cells in the hyaluronic acid hydrogel favored DCX-positive neuroblast formation 1 week after transplantation.[173] The study by Yuan et al. also observed the expression of nonmature neuronal markers of the engrafted cells such as beta tubulin and nestin.[164] Moreover, a low fraction of cells differentiated toward astrocytes, as shown by GFAP expression. Nonetheless, this study showed a preferential neuronal differentiation of engrafted iPSC-derived neuronal precursors after transplantation,[164] which is supported by the study of Jensen et al.[171]

To examine whether the transplanted iPSCs contributed to the synaptic network in the host brain, various methods to determine synaptic integration were applied. One method that was used to determine synaptic integration was by retrograde tracing of fluorogold in the ipsilateral globus pallidus, 9 weeks after the injection of iPSCs.[58] In this study by Oki et al., it was shown that a small fraction of the transplanted iPSCs were fluorogold positive,

med21400 W3G-med July 9, 2016 9:43

meaning that striatal transplanted iPSCs extended projections toward the globus pallidus. Other strategies to identify axonal projections of transplanted iPSCs used antibodies directed against donor specific cytoplasmic[57] or surface markers.[169] The study by Polentes et al. observed iPSC-derived axonal projections from the site of engraftment that was in the lesion cavity located in the lateral quadrant of the striatum to the striatum and globus pallidus after 1 month and these projections extended into the substantia nigra 1 month later. A few fibers were found in the corpus callosum.[169] The study by Tornero et al. that compared long-term expandable neuroepithelial-like stem cells and long-term expandable neuroepithelial-like stem cells fated toward cortical neurons observed a higher density of projections extending from the site of engraftment in the cortex over the corpus callosum in fated cells compared to the nonfated cells.[57] In addition to axonal projections, the engrafted cells were shown to be functionally active up to 6 months after transplantation, as was shown by whole-cell patch-clamp recordings in acute brain slice preparations.[57,58]

In addition to cell–replacement mechanisms and synaptic integration, paracrine-mediated improvement of stroke outcome is another mechanism by which transplanted iPSCs can exert their effect. The study by Oki et al. showed that functional recovery was independent of long-term engraftment, suggesting a beneficial effect early after transplantation.[58] Furthermore, they showed that VEGF reactivity was upregulated following transplantation in astrocytes and the blood vessel wall of the damaged brain as early as 1 week after transplantation. However, 9 weeks after transplantation, when animals receiving the cell graft showed significant functional improvement, no difference in vessel length density and immunoreactivity for the endothelial marker CD31 was observed between animals that were injected with iPSCs compared to vehicle-injected animals. An additional study by this research group detected only weak expression of VEGF in the grafted cells and blood vessel walls of the damaged brain 8 weeks posttransplantation. VEGF-expressing astrocytes were not observed.[170] This suggests that VEGF signaling is important in early recovery after stroke and can trigger long-lasting effects in brain plasticity. In addition, it can also be postulated that VEGF signaling alone is not sufficient to clarify the improved functional recovery after iPSC transplantation.[180] Moreover, Tatarishvili et al. observed functional improvement from 1 to 4 weeks after transplantation, making it inconceivable that the behavioral improvement is due to cell-mediated neuronal replacement.[170]

Another possible mechanism by which transplanted iPSCs can influence functional recovery after stroke is by modulating the immune response. Microglia were not found to be more prominent or more activated in vehicle-treated animals compared to animals that received the iPSC graft 8 weeks after transplantation. However, microglia in the vehicle-treated group showed a more round/amoeboid morphology compared to animals that received the iPSC graft.[170] There is no information on how the number and activation status of the microglia was affected in the early time points after transplantation. Previous studies have shown that NSCs, transplanted in the cortex or striatum after stroke, can reduce the number of microglia in both early as late time points after engraftment.[54,181] In addition to these results, Chen et al. previously reported an upregulation of antiinflammatory cytokines and a downregulation of proinflammatory cytokines after nonpredifferentiated iPSC administration in the stroke brain.[182] More recently, a study by Eckert et al. showed that intrahippocampal transplantation of iPSC-derived NSCs 24 hours after stroke onset reduced the expression of proinflammatory markers, microglial activation, and adhesion molecules while attenuating BBB damage, leading to a significant improvement in motor and sensorimotor function within the first week after transplantation. These data support the influence of early transplantation on the host immune response, leading to an improved stroke outcome.[172]

These results show that functional integration of transplanted iPSCs in the host model is achievable. However, the timing of graft-induced improvement in behavioral tests suggests

med21400 W3G-med July 9, 2016 9:43

that early amelioration of stroke symptoms is mediated by paracrine mechanisms and may be mediated by the early influence of the graft on the host immune system. In addition, functional improvement caused by the integration of graft-derived neuronal cells is expected to take longer than the 8–9 weeks follow-up in the presented studies. The presented studies by Oki et al.[58] and Tornero et al.[57] followed the engrafted cells for up to 6 months after transplantation and were able to observe neuronal differentiation and functionality of the cells, but did not examine the underlying mechanisms into further detail.

One of the major issues in determining the fate of the transplanted cells and determining the exact onset of functional recovery is that most studies described above used traditional histopathological techniques to determine the fate of the transplanted cells and to visualize possible methods of action of the transplanted cells. Therefore, as will be discussed next, novel techniques are being developed to allow real-time and longitudinal noninvasive imaging and tracking of the transplanted cells. Furthermore, additional imaging methods can be applied in the same animals to get real-time information on revascularization and reinnervation as will be discussed next.

4. CURRENT CHALLENGES AND FUTURE PROSPECTS OF STEM CELL BASED THERAPIES IN STROKE: VISUALIZING DONOR CELLS AND THE INJURED BRAIN

Traditional histopathological techniques can only be applied ex vivo. The most frequently used cell trackers include the use of GFP-transduced or BrdU-labeled stem cells in addition to antibodies directed against human-specific epitopes. However, it has been shown by Burns et al. that thymidine analogs such as BrdU may not be a suitable marker to track donor cells due to label transfer to phagocytizing cells.[183] Similar to cell tracking, morphological changes such as revascularization and reinnervation, evoked by the transplanted cells, are investigated by using tissue-specific antibodies or other ex vivo methods. Fortunately, noninvasive, quantifiable imaging methods have been developed to track the fate of the transplanted cells and evaluate the host environment, which can also be applied in humans. These methods will provide more insight into the exact timing of behavioral improvements following stem cell transplantation by correlating the presence or absence of the engrafted cells with morphological adaptations such as revascularization and reinnervation at the injured site.

A. Visualizing Engrafted Cells in the Injured Brain

Noninvasive imaging methods of donor cells are based on magnetic resonance imaging (MRI), positron emission tomography (PET), single photon emission computed tomography (SPECT), and optical methods such as bioluminescence imaging (BLI) and fluorescence imaging (FLI). The advantage of MRI compared to the other imaging methodologies is its high spatial resolution. However, PET-, SPECT-, BLI-, and FLI-based imaging hold the advantage of a higher sensitivity, although the last two are only sensitive in preclinical research. Prior to injection, donor cells can be labeled either directly or indirectly. For indirect labeling, imaging reporter genes are introduced into the host cells that encode for proteins or molecules that will lead to the accumulation of a specific substrate or ligand within the cells in which the reporter is expressed. Direct labeling involves the (stable) attachment or incorporation of reporter molecules into the cells after in vitro incubation (extensively reviewed in Ref. [184]). Direct pretransplantation donor cell labels such as superparamagnetic iron oxide (SPIO) particles[60,185–187] and radionuclides[144,145] have also been used to track donor cell fate, but as will be discussed below, are also subjected to several disadvantages.[188] The different labeling strategies are illustrated in Fig. 3.

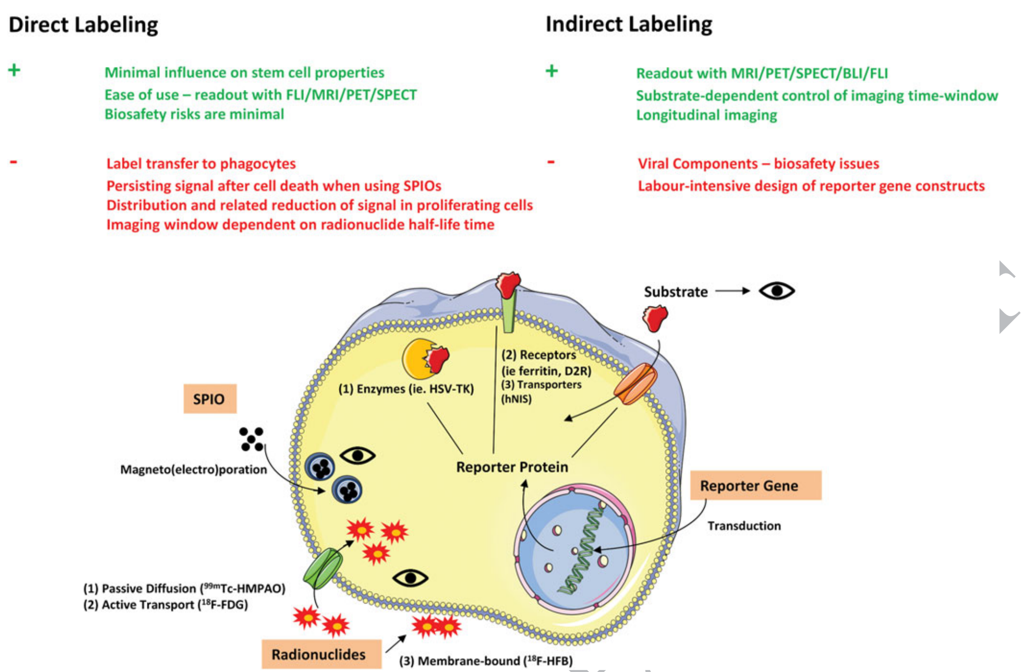

Figure 3. Direct and indirect labeling strategies to track stem cell fate in vivo. Direct labeling methods such as SPIOs and radionuclides have the advantage that they have been shown to have minimal effects on stem cell properties. Furthermore, biosafety risks are minimal and the labeled cells can be easily monitored with noninvasive imaging techniques such as FLI, MRI, PET, and SPECT. The disadvantages of using direct labeling strategies are the decreasing signal over time and the possibility of monitoring a nonspecific signal. These disadvantages can be circumvented by using indirect labeling methods in which reporter genes are incorporated into the donor cell genome of which the reporter protein can be visualized in a substrate-dependent manner, allowing spatiotemporal control of cell tracking. The major disadvantage of using this labeling strategy is the labor-intensive design of reporter gene constructs and biosafety issues regarding the use of viral components to transduce the donor cells. Image was created using Servier Medical Art.

SPIO particles are commonly used tracers that allow direct labeling of the donor cells for use with T2-weighted MR images without a significant effect on stem cell biology and differentiation potential of the labeled cells.[60,185–187] Despite the promising use of SPIOs in noninvasive cell tracking with MRI, this labeling method has several limitations.[189] It is impossible to discriminate between viable and dead cells as the SPIO particles remain present after the cells have died. Furthermore, macrophages and microglia present at the lesion sit may phagocytize the cell fragments of dead SPIO-labeled cells, which can lead to the occurrence of nonspecific signal not originating from transplanted cells. In proliferating cells, the SPIO signal decreases after transplantation, which is aggravated by asymmetrical replication of the donor cells.[189]

Another approach to directly label stem cells is with radionuclides that are detectable with the nuclear imaging methods PET or SPECT. These radionuclides can bind to the cell membrane (i.e., hexadecyl-4[^{18}F]fluorobenzoate)[190] or can be taken up by the cell via passive diffusion through the cell membrane or via ion channels, transporters, and pumps.[191–193] The most widely used PET-compatible radionuclide is 2-deoxy-2-^{18}F-fluoro-D-glucose (^{18}F-FDG). ^{18}F-FDG has been successfully used to monitor human autologous BMSC and peripheral hematopoietic stem cell homing after myocardial infarction in a human study.[194,195] Survival, proliferation, and differentiation of radiolabeled MSCs is maintained after radionuclide labeling, suggesting minimal radiotoxicity in these cells.[196–198] The two most frequently used SPECT-compatible radionuclides are oxine-bound Indium-111 (^{111}In-oxine) and technetium

med21400 W3G-med July 9, 2016 9:43

bound to hexamethylpropylene amine oxine (^{99m}Tc-HMPAO). ^{111}In-oxine and ^{99m}Tc-HMPAO have a half-life of 2.8 days and 6 hr, respectively, allowing long- and short-term imaging. ^{99m}Tc-HMPAO has been used as an effective tracer in a human study using labeled intraarterial-injected autologous bone marrow mononuclear cells after acute ischemic stroke[199] and shows minimal radiotoxic effects.[145] Although ^{111}In-oxine is an FDA-approved tracer and has been used in animal models of stroke and myocardial infarction[144,200] and human clinical studies,[201] it has been reported that ^{111}In-oxine binding is reversible, and that cell function and viability are impaired due to radiotoxic effects.[202,203]

Similar to SPIOs, direct labeling of stem cells with radionuclides also has several disadvantages, such as tracer leakage into the extracellular space that induces labeling of nontarget cells.[10,188] Furthermore, the tracer leakage to nontarget cells can be homogeneously distributed to these cells that leads only to a diminishable background signal compared to the initially targeted cells. More important is that the application window of these tracers is highly dependent on the half-life of the used radionuclide, reducing the use of these radionuclides for longitudinal follow-up of the labeled cells.[10] Radiotoxicity does not appear to be a major hurdle when using low doses of ^{18}F-FDG or ^{99m}Tc-HMPAO, but becomes a problem when ^{111}In-oxine is used as a tracer and is highly dependent on the dose that is used.[197]

To minimize the problem of label transfer from donor to host cells or to cope with the decreasing signal after transplantation of the donor cells, other noninvasive imaging methods are available. One of the possibilities is using reporter genes of which the proteins are only encoded in viable cells. Well-known examples are green fluorescent protein,[204] which can be detected by FLI, and firefly luciferase (*fluc*),[205] which catalyzes a light-emitting reaction that can be detected by BLI upon oxidation of exogenously delivered luciferin. BLI-compatible reporter genes have been successfully transduced in MSCs,[206,207] and iPSCs[156] and have been successfully used to monitor both donor cell fate[207] as endogenous NSCs.[208] Although these optical imaging methods provide a highly sensitive technique[209,210] to monitor cell survival and proliferation, these methods are limited by a loss of spatial resolution due to light scattering and by the anatomical depth of the signal-generating engrafted cells[211] making them only useful for preclinical research in small animals.

To improve the spatial resolution and to gain additional information of the engrafted cells, MRI-detectable reporter gene methods have been developed, which are listed in Patrick et al. (Table S1 in Ref. [212]) and reviewed in detail by Vandsburger et al.[211] MRI reporter genes are based on intracellular iron accumulation, enzymatic reactions, membrane-bound proteins such as the biotin–streptavidin interaction, and chemical exchange saturation transfer (CEST).[211] It should be noted that noninvasive MRI reporter gene methods that are based on visualizing iron accumulation cannot distinguish between living and dead cells, once the transduced cells have bound or accumulated iron.[211] Although MRI reporter genes based on cell membrane interactions and enzymatic reactions were developed, the most promising MRI reporter method is based on CEST. CEST is based on compounds containing exchangeable protons that resonate at a different frequency than bulk water protons. These protons can be selectively saturated with a radiofrequency pulse and are transferred to the bulk water molecules after proton transfer, attenuating the signal of the water signal.[213] However, to date, no stem cell tracking experiments have been performed with CEST.

In addition to MRI-detectable reporter genes, nuclear reporter genes that can be detected with PET or SPECT have been developed. These highly sensitive techniques allow for repeated visualization of migration and function of donor and host cells.[214] Imaging reporter genes can be subdivided into three main categories: enzymes, receptors, and transporters.[10]

The herpes simplex virus type 1 thymidine kinase gene (HSV1-*tk*) encodes the viral protein HSV1-TK, an enzyme that can phosphorylate nucleoside analogs that are subsequently negatively charged and become entrapped in the cells.[215] HSV1-TK can phosphorylate isotope-labeled pyrimidine analogs that can subsequently be used as reporter probes in PET and SPECT imaging.[191] Unfortunately, the use of viral proteins can potentially cause an immune response and more specific for stroke research, none of the HSV1-TK compatible tracers is able to cross the BBB.[216,217] These disadvantages of HSV1-TK compatible tracers can be overcome by using reporter genes encoding for receptors such as the dopamine D2 receptor (D2R)[218] and human somatostatin receptor subtype 2 (hSSTr2)[219] or by reporter genes coding for transporters such as the human sodium iodide symporter (hNIS) that can transport all radioactive forms of I^- as well as other isotopes (i.e., Technetium-99m).[220] The advantage of using D2R, hSSTr2, and hNIS is that they can be labeled with tracers able to cross the BBB and, since they are of human origin, are not likely to elicit an immune response.[191] However, D2R and hSSTr2 have not been used for longitudinal tracking of engrafted cells. hNIS has already been successfully used to track MSCs[221] and iPSCs[222] in vivo but to date, no studies have been performed to trace donor stem cells after transplantation in stroke with hNIS.

In order to combine the high spatial resolution and anatomical precision of MRI with the sensitivity of radionuclide or BLI, dual-modality probes have been developed to exploit the characteristics of each imaging method [212],[223] (reviewed in Ref. [224]). For example, Patrick et al. used Oatp1a1 as a reporter.[212] This molecule is able to mediate the cellular uptake of several small molecules, including gadolinium-based contrast agents that enhance T1-weighted images in MRI and the radionuclide ^{111}In, which can be detected with SPECT. Dual-modality imaging has also been used in stroke research to track transplanted NSCs[225–227] and MSCs.[143] In these studies, donor NSCs were transduced with *fluc* and labeled with SPIO particles to allow BLI and MRI imaging,[226,227] or were imaged with ^{19}F MRI after transduction with *fluc*.[225] In a study by Walczak et al., SPIO-labeled MSCs were monitored with MRI combined with laser Doppler flow.[143]

Over the past decade, several advances have been made in molecular imaging to facilitate stem cell tracking after transplantation. As will be discussed next, some of these methods are not only applicable to track the fate of the donor cells but can also be used to acquire additional information on the endogenous repair mechanisms following ischemic brain injury.

B. *In vivo Imaging of the Recovering Brain*

Similar to tracking the fate of donor cells, noninvasive imaging modalities based on MRI, radionuclide imaging, or optical methods can be applied to monitor the physiological and/or functional properties of the host environment. These methods allow visualization of neurovascular processes and neurological function but can also be used to study endogenous stem cells responses to treatment.

Optical methods are mainly based on FLI or BLI. In vivo two-photon FLI can be used to monitor blood flow, synapse formation, and neuroinflammation.[228–230] Two-photon imaging requires fluorophores, although label-free imaging has been successfully used to visualize the mouse brain.[231,232] FLI can also be used to directly monitor neuronal activity by using voltage sensitive dyes and proteins, which change their fluorescent properties in response to changes in transmembrane voltage.[233,234] Unfortunately, penetration depth is limited and a cranial window is required to visualize subcortical structures and repair processes.[233,235] BLI-based optical imaging methods are also limited by penetration depth, although it has been shown that the BLI signal can be detected through the intact skull.[208] BLI-based methods have been used to track endogenous neuronal stem cells and neurogenesis after stroke.[208] In addition, *fluc* under the control of the VEGFR2 receptor promoter has been used to evaluate poststroke angiogenesis with BLI[236] and when put under control of the toll-like receptor 2, the response of microglia could be observed after photothrombotic stroke.[237]

Another approach is to use PET or SPECT compatible radionuclides such as ^{18}F-FDG, which has been used in animal models of stroke to evaluate stroke outcome with PET after transplanting iPSCs and ESCs.[238] This study by Wang et al. was able to correlate the ^{18}F-FDG PET signal with functional improvement as shown by a decrease in mNSS score in animals that received a nonpredifferentiated iPSC or ESC graft. Although the donor cells were not predifferentiated, tumor formation was not observed up to 4 weeks posttransplantation. A similar study was performed by Daadi et al. where NSCs were labeled with SPIO particles and transduced with HSV1-*tk* to allow PET with the reporter probe [^{18}F]-FHBG prior to transplantation in a rat stroke model.[239] In accordance with the study of Wang et al., Daadi et al. demonstrated an increased ^{18}F-FDG PET signal after NSC transplantation and correlated the ^{18}F-FDG PET signal and the presence of NSCs as shown by [^{18}F]-FHBG detection. Increased glucose uptake might be indicative of enhanced neuronal function but caution needs to be taken when interpreting ^{18}F-FDG PET results as it is unclear which mechanisms are responsible for the increased ^{18}F-FDG uptake in the injured brain.[240] More recently, a study by Zinnhardt et al. combined a multitracer PET study with MRI to link the spatiotemporal relationship of MMP and microglial activation after transient MCAO providing more detailed information on early and delayed endogenous stroke responses.[241]

MR-based methods have the advantage that they can couple physiological information with anatomical characteristics of the region of interest. Therefore, several MR-based modalities are used to gain additional information of the host environment in stroke and will be discussed next.

Blood oxygenation level dependent functional MRI (BOLD-fMRI) depends on the hemodynamic response to neuronal activity, which are directly related to the energy demand of the studied brain areas.[242] In small animal stroke research, BOLD-fMRI after electric forepaw stimulation has been successfully used to assess functional recovery and electric brain activity.[243] As stated previously, current therapies for ischemic stroke aim to salvage the ischemic penumbra. Therefore, it is important to have an adequate monitoring tool to evaluate the size of the penumbra before and after therapeutic intervention. By using diffusion- and perfusion-weighted MRI, the size of the ischemic penumbra can be determined based on the area of diffusion/perfusion mismatch.[244] While the diffusion/perfusion ratio is mainly used clinically, it has also been successfully applied in a rat model of ischemic stroke.[245,246]

In addition to providing a ways of evaluating the size of the penumbra, also the architectural information of the affected brain region can be visualized with MR modalities. Diffuse tensor imaging (DTI) images the anisotropy of water molecules in different tissues and is mainly used in stroke research to study and visualize white matter tracts.[247] Although its use in experimental stroke in animal models is limited, DTI has been used to study the MRI evolution of stroke macaques.[248] While DTI can provide architectural information of white matter organization after stroke, manganese-enhanced MRI (MEMRI) can be used to image synaptic connectivity and assess changes in neuroarchitecture, anterograde axonal transport, and demarcates active regions of the brain independent of hemodynamic contrast compounds.[249,250] MEMRI uses T1 contrast-enhancing Mn^{2+} ions that can enter the cell via Ca^{2+} channels. As Ca^{2+} plays a crucial role in neuronal activation Mn^{2+} is transported toward the synaptic cleft, where it can be taken up by other neurons in the circuit.[251] Neuronal connectivity during stroke and recovery has been evaluated in MCAO models using this technique.[252,253]

Reperfusion plays a key role in repairing the ischemic lesion and can be examined with arterial spin labelling (ASL) scans in order to quantify the absolute amount of tissue perfusion in different brain areas evoked by therapeutic interventions, including stem cell based therapies.[254] ASL measurements in small animal models of ischemic stroke were able to visualize perfusion in the ischemic brain, allowing the potential longitudinal follow-up of therapeutic interventions that aim to enhance reperfusion.[255,256]

5. DISCUSSION AND PERSPECTIVES

Because of the promising preclinical results of stem cell based therapies in in vivo models of ischemic stroke, small-scale human trials were performed using IV delivered autologous MSCs.[257–259] The outcome of these studies showed that MSC transplantation improved the disease outcome but stress that the underlying mechanisms of action need to be determined to provide a more directed approach, although it was reported that the SDF-1 levels in the serum of MSC-transplanted patients were associated with the clinical outcome.[259] Therefore, it is important that in the preclinical phase the potential of stem cell based therapies is thoroughly investigated in animal models of ischemic stroke. Each model and route of administration has its own advantages to investigate specific mechanisms of action of the donor cells. IA- and IV-delivered donor cells would be the preferred method of administration in human applications, but this administration route requires a substantial amount of donor cells compared to intracranial delivered stem cells and are hindered by several limitations. These include high morbidity and cells ending up in the spleen after IA delivery[143,260] and pulmonary obstruction after IV delivery of donor cells.[145,261] However, several reports are available that state that donor cell migration toward the spleen is a possible mechanism of action of stem cell mediated regeneration following stroke. Acosta et al. demonstrated that IV-delivered BMSCs end up in the spleen, but that BMSC migration to the spleen inversely correlated with a reduced infarct size, periinfarct size, and the number of MHC-II positive activated cells in the striatum.[134] Although the influence of MSCs themselves on poststroke immunosuppression was not investigated, Doeppner et al. reported that poststroke immunosuppression was attenuated after MSC extracellular vesicles were injected in stroke mice.[129] Supporting this hypothesis, Vendrame et al. investigated the immunomodulatory effects of the mononuclear cell fraction of umbilical cord blood cells.[262] In this study, it was demonstrated that IV transplantation of these cells diminished spleen reduction and rescued $CD8^+$ T-cell counts in addition to a reduction in brain damage. Moreover, it was shown that the cell transplant increased Il-10 and interferon gamma mRNA expression and decreased tumor necrosis factor alfa mRNA expression.[262] Donor cells applied to the host circulation migrate toward and integrate in low numbers into the brain lesion and ameliorate the disease outcome (See Table I) presumably by neurotrophic effects as the cerebral level of neurotrophins was found to be elevated in some studies.[132,135,139] Moreover, IA or IV delivery of donor cells allows the interaction of the BBB with donor cells to be studied that can contribute to knowledge of the neuroimmunological response after ischemic stroke.[128] Intracranial delivery of donor cells is the most invasive route of transplantation but a thorough meta-analysis of preclinical data by Vu et al. showed that intracranial delivery of MSCs provided greater clinical benefit, although this mode of administration is less favorable for human applications due to the highly invasive nature of the transplantation procedure.[263] Nonetheless, intracranial transplantation of fetal nigral tissue to treat patients with Parkinson's disease has been performed although the clinical benefit remained debatable.[264]

Donor cells remain detectable at the site of injury and ameliorate the disease outcome as shown by behavioral results and postmortem tissue analysis. However, functional integration of the engrafted MSCs is based on marker expression instead of electrophysiological recordings. The latter has only been performed for iPSC-derived cortical differentiated long-term expandable neuroepithelial-like stem cells, demonstrating functional integration of the engrafted cells.[57,58] IA or IV delivery of iPSC-derived neuronal precursor or committed cells has not been performed to date.

When considering the most suitable source of stem cell as a potential therapy in stroke research, comparative studies are needed to highlight differences in therapeutic potential of stem cells from different sources in an analogous experimental setup. For example, studies comparing different subtypes of MSCs showed that ASCs are more suitable as a candidate

MSC than BMSCs[139] while another study compared the same subsets of MSC and could not observe any difference.[60] Moreover, the studies listed in Tables I and II describe both xenogenic transplantation of human-derived MSCs and iPSCs as allogeneic-derived MSCs in animal models for ischemic stroke. Studies that compare xenogenic and allogeneic MSCs in ischemic stroke are limited to the study by Yasuhara et al.[137] and Balseanu et al.,[149] who compared human and rat BMSCs, and Guttiérrez-Fernández et al. who compared human ASCs with rat-ASCs.[151] In the study by Yasuhara et al., it was shown that the allogeneic BMSC graft showed a higher survival rate and a higher number of neurogenic differentiated cells. In both engrafted groups, improvement in locomotor and neurological function and a reduced loss of striatal periinfarct cells was observed.[137] Although Balseanu et al. used both rat and human BMSC in their study, no direct comparison was made between the xenograft and allograft.[149] Preclinical studies with autologous-derived stem cells are scarce and were only described in Jiang et al.'s[131] study who transplanted autologous rat ASCs in ischemic rats, whereas for other subtypes of MSCs, preclinical studies with autologous stem cells are not available. This study by Jiang et al. did not compare the outcome of the transplantation between autologous and allogeneic ASCs, which would provide additional information on extrapolability of their results. Although xenogenic stem cell transplants are not likely to be used in human studies, preclinical studies using xenogenic grafts provide insight into the potential of human stem cell sources in ischemic stroke.

To date, no studies have been performed that compare the therapeutic potential of iPSCs with a subtype of MSCs. Although the most thoroughly studied stem cell type in stroke research are MSCs, the potential of iPSCs and iPSC-derived neural progenitor cells is currently being intensively investigated with several very promising results when iPSCs are predifferentiated toward neuronal precursor cells or committed cortical neurons.[57,58] Nonetheless, adequate screening of fully neuronal committed cells preceding engraftment is recommended for several reasons. As stated previously, a recent study by Choi et al. demonstrated the ability of the iPSC-derived NPCs to return toward their pluripotent and thus tumorigenic state by transgene Q7 reactivation during differentiation.[157] This statement was supported by Liu et al.[156] and Fu et al.[158] who demonstrated that nondifferentiated iPSCs remain present in an iPSC-derived progenitor pool. Another problem with iPSCs, and more specifically with retroviral transduced iPSCs, is the retroviral gene integration in the host, which promotes tumorigenicity.[265] Therefore, additional approaches have been developed to generate iPSCs with a lower risk for tumorigenicity.[266,267] Moreover, it has been shown that iPSCs retain an epigenetic memory related to the somatic donor tissue, leading to spontaneous redifferentiation to the cells of the tissue of origin,[268–270] although it has been shown that this epigenetic memory and redifferentiation rate can vary between the somatic cells of origin with different tumorigenic propensities between the somatic donor cells.[269,270] The tumorigenicity of human iPSCs (and ESCs) was thoroughly reviewed by Ben-David and Benvenisty.[271] As an alternative to iPSCs in which the Yamanaka factors are transduced, exogenous gene-free iPSCs can be used.[272] Another option is to additionally engineer iPSC-derived cells to express suicide genes to eradicate the cells, an approach that was successfully used in ESCs and BMSCs.[273,274]

It should be noted that in addition to various MSC sources and iPSCs, encouraging results have been achieved by using BMMNCs in animal models of ischemic stroke where this subset of cells was found to stimulate endogenous angiogenesis[61,62] and neurogenesis by improving the NPC-vascular niche[63] or modulate the immune system.[95] Moreover, it was shown that after ischemic stroke, the amount of $CD34^+$ blood cells that migrate from the bone marrow to peripheral blood is increased, which is associated with a better clinical outcome and is believed to be mediated by granulocyte colony-stimulating factor (G-CSF).[275,276] In addition to its effect on BMMNC recruitment, this factor has previously been shown to possess neuroprotective and neuroregenerative effects[277] and is also believed to mobilize BMSCs and possess immunomodulatory properties.[278] BMMNCs can also be quickly isolated from peripheral blood by Ficoll-Paque density gradient centrifugation just before administration, circumventing the additional cell culture period, which is required for MSC- or iPSC-based therapies.[63] As was a hurdle with MSC-based therapy, also the administration route of BMMNCs remains a topic of debate. Although study by Kamiya et al.[96] showed superior results after IA-delivered BMMNCs over IV-delivered cells, this was contradicted by Yang et al. who showed that IA delivery was not superior to IV-delivered BMMNCs.[279] Subsequently, these cells have been used in several clinical trials. IA administration of these cells was safe and showed an improved clinical outcome.[280,281] IV delivery of these cells also appeared safe,[282] although no clinical improvement was observed in the study by Prasad et al.,[283] whereas Savitz et al. were able to show an improved functional outcome.[282] Despite these encouraging results, in depth in vitro evidence on the underlying mechanisms of action is largely unknown. The scarce in vitro data on the effect of BMMNCs on regenerative processes showed that BMMNCs exerted protective effects on rat hippocampal brain slices subjected to oxygen and glucose deprivation[98] and that the BMMNC secretome induced neuronal differentiation of SH-SY5Y neuroblastoma cells.[97] Nonetheless, additional in vitro data supporting the mechanism of action of BMMNC-based therapy for ischemic stroke are required.

Q8

As stroke is a disease that mainly affects the elderly,[7] it is important to take into account the effect of the aged microenvironment, age-related comorbidities, and the aged immune system on the outcome of stem cell based therapies.[230,284,285] Although it does not appear from clinical studies that the aged brain microenvironment is detrimental for stem cell based therapies, differences exist between the young and aged brain. For example, the formation of the glial scar is accelerated after stroke that hinders functional repair in aged rats.[286] Moreover, comorbidities such as hypertension, hyperlipidemia, and diabetes mellitus appear to play a role in age-related stroke severity.[287,288] As described previously, angiogenesis is a key concept in establishing brain repair. Buga et al. compared the transcriptome and immunochemistry of young and aged stroke rats and poststroke patients. Remarkably, although the upregulation of proangiogenic genes associated with processes such as vessel sprouting, tube formation, and maturation was delayed in aged rats, angiogenesis in the aged brains was similar to their younger counterparts. In addition, an upregulation of proinflammatory and scar-promoting genes was found in the aged rats compared to the younger brains, supporting the accelerated scar formation and increased neuroinflammation in aged stroke subjects.[289] Of the studies listed in Table I, two studies by Shen et al.[146,147] and studies by Taguchi et al.,[152] Balseanu et al.,[149] and Zhang et al.[148] used aged rats to perform MSC transplantation studies in ischemic stroke. Although these studies did not directly compare the outcome of their transplantation study between young and aged rats, several encouraging results were found in these studies. These included, but are not limited to, enhanced functional recovery,[146,148,149,152] a reduction of the glial scar thickness,[146,147] and improved angiogenesis.[148,149,152] Remarkably, Balseanu et al. who used a G-CSF treatment, which is believed to possess multiple regenerative effects,[278] or a combination of G-CSF and a single BM-MSCs dose to improve the functional outcome in aged stroke animals, observed that the functional improvement was not increased in this combination treatment.[149] Similarly, Buga et al. who used a G-CSF and a combined G-CSF–BMMNC therapy to improve the functional outcome after transplantation in aged stroke animals were also unable to observe an increased functional improvement by using the combination treatment.[290] The use of iPSCs in aged rats was described by Tatarishivili et al. who showed that almost 50% of the engrafted iPSC-derived long-term expendable neuroepithelial cells survived 8 weeks post-transplantation and caused functional improvement in the aged rats.[170] Moreover, these cells differentiated toward neuroblast-like cells, compared to the BM-MSCs described in Refs. 146 and [147] where the few surviving cells predominantly differentiated toward astrocytes. These studies support the use of aged animals for in vivo stroke studies in which they were able to observe cell-mediated improvements in brain regeneration and functional recovery. Nonetheless, a direct comparison of stroke outcome and the molecular effect after cell transplantation between young and aged animals for the various age-related differences in (brain) microenvironment could provide additional information on which processes are mainly responsible for stem cell mediated brain repair. For example, the previously mentioned study by Buga et al., which described the transcriptome in young and aged rats after stroke, provided several new target pathways that can provide additional insight into age-related stroke pathology and therapeutic opportunities.[289]

The evaluation of the disease outcome in animal models for ischemic stroke is based on behavioral testing, for which various tests can be applied to investigate specific aspects of neuronal recovery.[126] As stroke symptoms are largely dependent on the brain area involved, different behavioral tests are applied to evaluate general, motor, sensorimotor, and cognitive recovery and each test has its strengths and weaknesses. For example, the Bederson test is easy to perform but reliable neurological ratings on this Bederson scale are limited because of their subjective nature, a common feature of all behavioral tests that are based on human observation. An overview with critical comments on the different behavioral tests that are often used in stroke research is provided by Schaar et al.[126] Nonetheless, functional assessment of stroke outcome should include tests to cover all aspects of the disease outcome.

As indicated above, preclinical stroke research is facing several important issues that need to be resolved prior to more elaborate testing of the clinical potential of stem cell based therapies for ischemic stroke. Noninvasive imaging methods can aid in the longitudinal follow-up of stem cell fate and effect after transplantation via various administration routes. However, with the exception of a few studies,[238,253] most of the studies that used noninvasive imaging to monitor the poststroke microenvironment or stem cell migratory pathways focused on the proof of principle of the imaging technique and did not link their results to functional recovery,[206,208,236,246,256] which will most likely be the next step to be performed with these highly promising imaging modalities.

6. *CONCLUSION*

Although multiple clinical advances have been made to improve the clinical diagnosis and outcome after acute ischemic stroke, beneficial long-term or delayed interventions are currently not available. Stem cell based therapies with MSCs and iPSCs have shown great promise in in vivo models of ischemic stroke through various administration routes. Nonetheless, the mechanisms of action of the transplanted cells remain poorly understood and are highly dependent on administration route, pretreatment, and full neuronal predifferentiation when using iPSCs. Although postmortem cell tracking methods provide detailed spatial information on the donor cell fate and host microenvironment, they are unable to deliver dynamic information on these subjects. A longitudinal follow-up with noninvasive imaging methods allows donor cell fate and changes in host microenvironment to be linked with behavioral and functional improvements, which can lead to additional insight into the mechanisms responsible for functional recovery in stroke after donor cell transplantation.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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