Regeneration after stroke: Stem cell transplantation and trophic factors

Monica Chau, James Zhang, Ling Wei, Shan Ping Yu

Abstract:

Stroke is a leading cause of death and disability worldwide. However, there is only one Food and Drug Administration-approved drug for the treatment of ischemic stroke, i.e., tissue plasminogen activator, and its therapeutic window is limited to within 4.5 h after stroke. Since clinical trials for neuroprotection have failed to demonstrate efficacy, multipotent and pluripotent stem cell transplantations are viable candidates for stroke treatment by providing trophic factor support and/or cell replacement following injury. The goal of this review is to highlight the promise of stem cell transplantation as vehicles for trophic factor delivery. The beneficial effects of different stem cell types as transplants as well as ways to upregulate trophic factors in stem cells are described in this review. Stem cell transplantation has consistently shown beneficial effects in the ischemic stroke model, in part due to the beneficial factors that stem cells release around the stroke injury area, resulting in smaller infarct volumes and regeneration and functional recovery. Upregulation of beneficial factors in stem cells and neural progenitors before transplantation has been shown to be even more effective in treating the stroke injury than stem cells without upregulated factors. However, for both stem cells and genetic engineering, there remain many unanswered questions and potential for improvement. These include modifiable parameters such as the different stem cell types and different factors, as well as the various readouts for investigation, such as various *in vivo* effects, such as immune system modulation and enhancement of endogenous neurogenesis and angiogenesis.

Key words:

Regeneration after stroke, stem cell transplantation, trophic factors

Introduction

Ischemic stroke

Stroke is a leading cause of death and disability in the United States and worldwide.[1] Of all strokes, 87% are ischemic in nature and 13% are hemorrhagic.[1] Approximately 795,000 individuals per year experience a stroke in the US, which can result in death or disability.[1] The risk factors that are most commonly associated with ischemic stroke are hypertension, diabetes, and smoking, which are all conditions that are highly prevalent worldwide.[2] Ischemic stroke occurs when a blood vessel is occluded in the brain, resulting in the loss of glucose, oxygen, and nutrients. This insult to the brain tissue either compromises or abolishes the function of that brain area. Due to the occluded blood vessel, cells in the area stereotypically undergo two waves of cell death.[3] First, a wave of rapid cell death occurs within minutes to hours of ischemia. Unless the clot is disintegrated with a thrombolytic drug, a later delayed wave of cell death occurs from hours to days after a stroke event spreading to areas further from the initial blockage. Stroke researchers are most interested in preserving the cells that are involved in the second wave of cell death because these cells possess greater salvage potential with treatment. This review will focus on the benefits of stem cell transplantation and its trophic factors for regenerative treatments of ischemic stroke.

Current stroke treatments are limited

With the growth of the obese and aged demographics, leading to increased incidence hypertension and diabetes, an ever-burgeoning population will be at risk for stroke. Even with the current demand for treatment due to the prevalence of stroke and its financial strain on health care, the only Food and Drug Administration (FDA)-approved drug available for stroke patients is tissue plasminogen activator (tPA). tPA is a thrombolytic agent that can break up clots to allow blood flow to return. Even with its great efficacy, tPA use is limited because it is quite time-sensitive, allowing only a narrow therapeutic window for its administration – within 4.5 h after stroke.[4]

Department of Anesthesiology, Emory University School of Medicine, Atlanta, GA 30322, USA

Address for correspondence:
Dr. Shan Ping Yu,
Department of Anesthesiology, Emory University School of Medicine, Atlanta, GA 30322, USA.
E-mail: spyu@emory.edu

How to cite this article: Chau M, Zhang J, Wei L, Yu SP. Regeneration after stroke: Stem cell transplantation and trophic factors. Brain Circ 2016;2:86-94.

Administering the drug outside of the recommended window increases the risk of intracerebral hemorrhages exacerbating the injury. A tPA safety study indicates that even with appropriate administration within 3 h, the symptomatic intracerebral hemorrhage rate was 5.2%.[5] Although tPA is efficacious when administered in the appropriate timeframe, only a small percentage of stroke patients actually receive the drug often due to the delayed arrival of the patient to the hospital and/or delayed diagnoses. Even though this drug is commonly used, there is no treatment that stimulates the regeneration of tissue after it is lost from stroke if the patient does not receive tPA in time. Another notable treatment is mechanical revascularization to surgically remove the thrombus or embolus.[6] While clinical trials have shown that these FDA-approved surgical devices are effective in improving 3-month functional outcomes and reducing 3-month mortality rates, there remain high rates of futile recanalization and hemorrhagic complications, especially among older patients, which is the majority of stroke patients.[7,8] Thus, there is a great unmet clinical need for regenerative treatments for those who have suffered a stroke.

The field of stroke therapy has been replete with researchers investigating a broad range of treatments. Of note, there have been numerous studies testing pharmacological neuroprotective agents that have been successful in laboratory stroke models but have failed in clinical trials. One representative agent is erythropoietin (EPO), a hematopoietic hormone, first used as a therapy to stimulate red blood cell generation as a treatment for anemic patients.[9] It has been used safely for this purpose for two decades now, is well-tolerated, and is commonly used to treat anemia. Numerous laboratory studies have championed the neuroprotective effects of EPO in stroke models.[10] However, its limited benefits in clinical stroke studies have engendered thromboembolic complications[11] and have also raised doubts as to whether or not EPO has any significant benefit on stroke.

Stem cell transplantation has been considered a promising avenue for the treatment of stroke.[12,13] Stem cells offer two primary mechanisms of treatment. One mechanism is for the cells to differentiate and regenerate the tissue lost from stroke. Another mechanism is that the stem cells themselves are vehicles of trophic factor delivery, thus encouraging endogenous regeneration and neuroprotection after stroke. The actions of stem cell regeneration are indeed 2-fold: Cell replacement and trophic factor release. Endogenous repair, attenuation of inflammation, and neuroprotection can be maximized by the trophic factors naturally secreted by stem cells.[14,15] These therapeutic approaches may preserve neuronal function and decrease the disability of stroke sufferers. This review will highlight the benefits of stem cell transplantation, the effects that their trophic factors can have on stroke tissue and brain function, and how to upregulate these factors before transplantation.

Stem Cell Transplantation for Stroke

The field of stem cell transplantation encompasses a wide range of cell types, including pluripotent and multipotent stem cell types. Stem cell types that have been tested in stroke models include hematopoietic stem cells, mesenchymal stem cells (MSCs),[16] embryonic stem (ES) cells,[17] neural progenitors,[18] multilineage-differentiating stress-enduring cells,[19] and induced pluripotent stem (iPS) cells.[20-23] Stem cell transplantation has demonstrated a multitude of beneficial effects such as increasing functional recovery, angiogenesis, and neurogenesis.[24] Furthermore, the benefits of stem cell transplantation are not limited by their route of administration, with multiple modalities demonstrating functional benefits including intracranial,[13] intravascular,[25] and even intranasal[26,27] delivery of cells. Our understanding of the benefits of stem cell transplantation still expands with the exploration into the nuances of their multifactorial effects including trophic factor release.

Stem cell types
Mesenchymal stem cells
MSCs are a multipotent stem cell type and are an attractive cell type because they can be harvested and transplanted autologously (i.e., from the same individual). MSCs are very clinically relevant as they are already being used in transplantation for diseases such as leukemia, lymphoma, immunodeficiency disorders, and severe anemia. Depending on the source of the stem cells, transplantation of MSCs can be either syngeneic or allogeneic. Syngeneic (i.e., possessing the same genetic information) MSC transplantation using autologous cells derived from the host has its benefits in circumventing immune rejection and avoiding complications such graft-versus-host disease. This cell type, particularly bone marrow mesenchymal stem cells (BMSCs), has been commonly studied in the laboratory through transplantation into stroke models[26,28] and has also been studied in small clinical trials.[29-31] While MSCs are multipotent, they are not pluripotent cells and thus have a limited neuronal differentiation potency. Whereas pluripotent cells can differentiate into any cell type in the body, MSCs are multipotent and are committed to differentiating into certain cell types such as stromal cells, osteoblasts, and chondrocytes.[32] Despite this limited potency, studies have demonstrated that MSCs can be differentiated into neural lineage cells such as neurons and astrocytes, but the differentiation efficiency tends to be low.[33] Nonetheless, MSC transplantation provides benefits aside from purely cell replacement and has demonstrated therapeutic effects after stroke. For example, transplantation of BMSC has reduced infarct size in stroke models, most likely due to a trophic factor effect.[26] Furthermore, MSC transplantation has resulted in increased endogenous cell proliferation and functional recovery.[26,34]

Recently, MSC transplantation has progressed beyond the laboratory and been translated into the clinical setting for stroke. Small clinical trials using MSCs have demonstrated that MSC transplantation improves functional outcomes in patients.[29-31] Lee *et al*. conducted a clinical trial comprising 16 stroke and 36 control patients to evaluate intravenous injections of MSCs following ischemic stroke.[30] Autologous MSCs were aspirated from patients' bone marrows before they were introduced back into circulation for systemic dissemination and trophic factor release, including to infarct regions. A follow-up study was performed at 5 years after transplantation. Importantly, the MSCs did not cause adverse effects, and compared to control patients, the MSC patient group had a better functional outcome according to a follow-up modified Rankin scale score.

Of note, there was also a correlation between outcome and the trophic factor, stromal derived factor-1α, measured from the patient serum 1 year after MSC treatment, although the sample size was too small for a conclusive causation effect to be drawn.[30]

Embryonic stem cells

Unlike MSCs, ES cells are pluripotent and have the potential to differentiate into any cell type of the body. ES cells are harvested from the inner cell mass of a blastocyst while the epiblast cells are still in their developmental naive (or ground) state.[35] They are good candidates for cell replacement since they can differentiate down the neural lineage into any of the three neuronal types: Neurons, oligodendrocytes, and glia. Numerous studies have evaluated neural progenitors derived from these cells after they are transplanted into stroke animals. Increasing evidence illustrates that these cells can undergo neuronal differentiation, promote regeneration, and increase functional recovery.[18,36] Nonetheless, these cells remain controversial for clinical implementation due to ethical issues and concerns surrounding the use of human embryos. Unlike MSC transplantation, transplantation of ES cells would not be autologous. With allogeneic (i.e., possessing nonsimilar genetic information) cell grafting, there is a risk of immune rejection. Furthermore, there is a risk for tumorigenesis with pluripotent cells, especially in the undifferentiated state.[37] If cells are transplanted as pluripotent cells without differentiation down a certain lineage, there is a much greater likelihood of teratoma formation or tumorigenicity.[38] For this reason, pluripotent cells are frequently directed into a less potent primed state prior to transplantation that promotes differentiation into the target cell type and minimizes tumorigenesis. Indeed, before transplantations for stroke, pluripotent ES cells are predifferentiated down a neural lineage into neural progenitor cells (NPCs) for transplantation.[36,39,40] After transplantation, the NPCs will fully differentiate into postmitotic mature neurons in the brain parenchyma.[40,41]

Much work has demonstrated the capacity of ES cell-derived neural progenitors to differentiate into the entire gamut of neural subtypes including forebrain,[42] midbrain,[43,44] hindbrain,[45] and motor[46] neurons. Although we know the differentiation products, more characterizations are needed to understand their functional activities and the network connectivity of these differentiated neurons. In other words, what are these newly formed neurons doing and what other neurons are they talking to? Can they establish circuitry with the existing endogenous network? The paragon of stroke regeneration would be a repair at cellular and pathway/structural level. This would require the exogenous cells to organize in the same way that the brain architecture was organized before the injury, including establishing the original circuit connections, synaptic connections, and neuronal-glial interactions in the neurovascular unit. Furthermore, the exogenous cells must behave with similar neurophysiology as before, including possessing similar firing patterns and action potential thresholds. Given the complexity of the system and the pathophysiology, there remains much ground to be covered in reaching this ambitious objective.

With the unrelenting advances in investigative techniques, however, this goal seems to be much closer in sight. Indeed, some researchers have already begun addressing some of these very interesting questions. For example, we demonstrated using electrophysiological recordings that 6 weeks after transplantation of bone marrow mesenchymal stem cells (BMSCs) into the ischemic cortex, the ischemia-disrupted intracortical activity from layer 4 to layer 2/3 was noticeably recovered, and the thalamocortical circuit connection was also partially restored. BMSC transplantation also promoted directional migration and survival of doublecortin-positive neuroblasts in the peri-infarct region. The investigation supported that BMSC transplantation has the potential to repair the ischemia-damaged neural networks and restore lost neuronal connections.[47] In another study, it was shown that ES cells transplanted into the focal ischemic sites terminally differentiated into neurons and glia cells. Subsequent immunofluorescence revealed that a subset of the grafted cells in the distal cortex had taken up the retrograde tracer Fluorogold 28 days after the transplantation.[40] This indicates that transplanted cells do have the ability to form connections and synapse onto other brain structures after transplantation, including reestablishing connectivity that had existed before the injury. These new measures of transplantation success, such as graft network connectivity, have enriched the field beyond testing the infarct size and functional recovery of the transplanted animal. With these higher levels of evaluations, we can begin to analyze the circuit-wide behaviors of the grafted cells, determine the factors that direct them to extend functional connections, and exploit these factors to promote graft-host cell engagement.

There are variations upon stem cell transplantation tested to optimize graft survival and regeneration. Some research groups have tested scaffolds, such as Matrigel or hydrogel matrix, to provide physical support to the transplanted cells within the brain parenchyma.[48-50] The scaffold can provide a favorable environment for the stem cell graft and also allow cells to reside in the stroke cavity of where the necrotic tissue had been. In one study, the biopolymer hydrogel composed of cross-linked hyaluronan and heparan sulfate promoted graft cell survival and reduced inflammatory cell infiltration to the cell graft.[48] Bioengineering a microenvironment to maximize stem cell survival and tissue regeneration has compelled the field to examine other ways to vary stem cell transplantation, such as genetic engineering and preconditioning strategies.[51]

Induced pluripotent stem cells

An emerging cell type that has been transplanted in stroke animals is iPS cells. These cells have emerged as a more translationally feasible option compared to their counterparts because iPS cells possess the advantages of both ES cells and MSCs. Like ES cells, iPS cells have an advantage as pluripotent cells for differentiation. Similar to MSCs, however, they can also be derived and transplanted autologously to avoid immune rejection. In 2012, Yamanaka received the Nobel Prize for his work in the creation of iPS cells. Yamanaka's group initially derived iPS cells from mouse fibroblasts in 2006[52] and then later from human fibroblasts in 2007.[20] iPS cells are adult somatic cells that undergo the upregulation of four genes that are required maintain pluripotency – Oct-3/4, Sox2, Klf4, and c-myc and are genetically reverted to the pluripotent stage.[20] These cells are similar to ES cells in that they can differentiate into cell types of the three embryonic germ layers; however,

unlike ES cells, they can be derived autologously from one's own somatic cells. For transplantation into the brain, iPS cells can be differentiated down a neural lineage into neuronal progenitors and neurons.[22,53] This technology is particularly amenable for therapeutic applications since patients would have access to a large reservoir of potential autologous stem cells generated from adult tissue. Since the cell transplantation would be syngeneic, the use of iPS cells would minimize immune rejection issues. Furthermore, because the cells are not derived from blastomeres, iPS cells would circumvent the ethical and political controversy surrounding embryonic cells.

A few groups have already studied the effects of transplanted iPS cells in stroke injury models. One of the first studies of this nature employed a focal ischemic stroke model.[22] They created their own iPS cells from mouse embryonic fibroblasts, with the transduction of the four transcription factors: Oct-4, Sox2, c-Myc, and Klf4 using retroviruses. They demonstrated that they were able to differentiate these cells into neurons with the expression of neurofilament and Class III β-tubulin (TUJ1), mature neuronal markers. Pluripotent cells were differentiated into neural progenitors, mixed with fibrin glue, and transplanted into the cortex preceding the stroke induction. Animals with iPS cell transplantation, both with and without the fibrin glue, showed a decrease in infarct volume.[22] When testing functional recovery with the rotarod and grasping strength assays after transplantation and stroke, animals with iPS cells and glue had the best functional recovery at 1, 2, and 4 weeks after stroke induction.[22] Similar to the aforementioned study with hydrogel, this study used fibrin glue in a similar way to provide a scaffold for transplanted cells.

Recently, another group was able to perform a similar study demonstrating the beneficial effects of iPS cells in stroke.[54] This study differs from the previous in that human iPS cells were transplanted and not mouse cells. Human iPS cells were generated from human fibroblasts and transplanted into rats with a middle cerebral artery occlusion (MCAO). Whereas Yamanaka's group showed reprogramming with Oct-4, Sox2, c-Myc, and Klf4,[20] later studies used a different set of factors, namely, Oct-4, Sox2, Nanog, and Lin-28 to successfully generate human iPS cells.[55] These cells were injected the cortical penumbra region of rats that underwent a 70-min filament MCAO. The injected cells were able to differentiate into neurons *in vivo* after the injection. Rats with iPS cell injection had a reduced lesion size and improved sensorimotor function.[54] Similarly, the transplantation of human iPS cells into a stroke mouse model has been shown to form functional neurons and increase functional behavior in animals with transplantation.[56] The cells were differentiated into neuroepithelial-like stem cells and exhibited neuronal functionality via electrophysiology. Importantly, mice transplanted with human iPS cells showed a functional recovery after stroke as assessed using the staircase behavior test.[56]

iPS cells are an especially promising therapeutic modality for stroke injury since they can be derived and transplanted autologously and can differentiate into any cell type, but there are still many optimizations and risks to be evaluated before translating this treatment into humans. For instance, iPS cells, similar to other pluripotent cell types, have the possibility for tumorigenesis after transplantation although that possibility is greatly reduced by committing the pluripotent cells toward a particular lineage before transplantation. Further, the proper dosage of cell delivery and age of cells used as well as the timing of the delivery must be optimized. The generation of iPS cells from host tissue requires a significant amount of time and will require careful coordination and execution if the cells are to be transplanted at a target time with sufficient yield after a stroke. The current research is also focused on improving upon existing techniques, such as using rotary cultures or preconditioning strategies,[18,51,57-59] to maximize the yield of differentiated products.

Stem cells as vehicles for trophic factor delivery

Stem cell transplantation may act alternatively to provide trophic factors for regeneration after injury. The importance of trophic factors to neuroregeneration and plasticity is instantiated by the fact that in some cases, the lack of or withdrawal of a trophic factor such as nerve growth factor (NGF) can trigger cell death.[60] Stem cells naturally express factors including vascular endothelial growth factor (VEGF), fibroblast growth factor, brain-derived neurotrophic factor (BDNF), and EPO that encourage repair. The idea that stem cells are effective vehicles and secretors of trophic factors is further supported by studies that injected BMSC-conditioned media into a stroke brain and led to functional benefits.[61] BMSC-conditioned media can recapitulate some effects of the cell transplantation itself. BMSC-conditioned media have been reported to increase neurite outgrowth, increasing neurite length and branch number in Ntera-2 neurons, supporting BMSC-associated paracrine effects.[62] The pleiotropic actions and benefits provided by stem cells are evident in less potent stem cell types as well. For example, intravenous injection of conditioned media derived from adipose stem cells conferred multiple regenerative and cytoprotective effects, including increased neovascularization, decreased neuronal and microglial cell death, and improved motor function following stroke.[63] Furthermore, infusion of trophic factors themselves, such as granulocyte colony-stimulating factor (G-CSF), has been shown to provide neuroprotective, angiogenesis, and neurogenesis effects after stroke and can even extend the therapeutic window for tPA administration.[64] MSCs release a wide range of adaptive factors, including factors that are involved in cytoprotection (endothelin), angiogenesis (VEGF, Smad4, Smad7), and cell migration (LRP-1, LRP-6).[65] In this review, we will focus on two major benefits of trophic support after stroke, angiogenesis and neuroprotection.

Trophic factors from stem cells increase angiogenesis

In order for stem cell transplantation to improve upon a stroke injury, several reparative events must take place. One of the major events of tissue regeneration involves rebuilding the vasculature, particularly the neurovascular unit of the stroke injury.[66] A major trophic factor is VEGF which is a major activator of angiogenesis, and administration of VEGF increases neovascularization and functional recovery after stroke.[67] In particular, VEGF stimulates the tubule formation of endothelial cells within an *in vitro* model (human umbilical vein endothelial cells) to increase vessel numbers.[68] The effect is attenuated with the addition of a VEGF inhibitor.[68] In an *in vivo* example of stem cell transplantation, VEGF secreted from neural stem cells increased neovascularization and attenuation of inflammation in the penumbra.[69] In animals

that received neural stem cell transplantation in the penumbra, there was a greater blood vessel density compared to naive and control animals at 2 weeks posttransplantation. Further, animals with neural stem cell transplantation demonstrated enhanced angiogenic signaling pathways exhibiting greater levels of phosphor-VEGFR2, Tie-2, and both cognate receptors for ligands: VEGF and angiopoietin 1 and 2. Increase in the phosphorylated form of VEGF indicates an increased level of signaling through the receptor in animals with cell transplant.[69] Neuroepithelial-like stem cells derived from human iPS cells express VEGF.[56] Their transplantation into the stroke mouse brain also shows increased levels of VEGF in astrocytes and blood vessels within the area surrounding the graft. However, interestingly, in this specific study, they were not able to find increased angiogenesis associated with the greater VEGF levels. This suggests that while not enough VEGF was secreted by the transplanted cells to cause an angiogenic effect, VEGF possibly provides an additional nonangiogenic role in the improved functional recovery that was observed. In all, the contribution of VEGF and stem cells to angiogenesis after the stroke site can be measured and evaluated by several methods: Blood vessel density, Western blot of angiogenic receptors, functional recovery, local cerebral blood flow[70] or vessel, and BrdU co-label in immunohistochemistry.

Trophic factors mediate stem cell effects on neuroprotective and neurogenesis

Stem cells can provide cell replacement as well as neuroprotection via trophic factor secretion. Although VEGF has mainly been studied in the context of angiogenesis, VEGF plays a role as a neuroprotective as well as neurogenic factor. Mice overexpressing VEGF had fewer neurological deficits and smaller infarct volumes than mice without overexpression.[71] Trophic factors can prevent cell death through their intersection with apoptosis pathways. There is evidence that VEGF prevents apoptosis by inhibiting the expression of pro-apoptotic genes, such as p53 and caspases, through its binding and activation of VEGFR-1, one subtype of VEGF receptors.[72] VEGF has also been shown to promote neurogenesis in the subventricular zone (SVZ) and subgranular zone, as well as endogenous migration of neural progenitors from the SVZ.[73] Thus, VEGF has neurogenic, neuroprotective, and angiogenic abilities. BDNF is another trophic factor released by stem cells that have been shown to provide neurotrophic properties and play a role

in neurogenesis.[74,75] BDNF intersects with apoptotic pathways to prevent cell death as evidenced in cerebellar neurons treated with BDNF.[41] There was more survival with granule neurons treated with 50 ng/ml of BDNF compared to cells with no treatment.[41] These trophic factors naturally expressed and secreted by stem cells have pleiotropic beneficial effects.

One-way that stem cell transplantation can be protective is through the reduction of inflammation. VEGF is a cytokine, and conventionally, it has been considered to be a proinflammatory factor.[76] This factor is paradoxical in its actions, in that it has been considered proinflammatory as well as adaptive and neovascularizing factor. When neural stem cells were transplanted into the peri-infarct area, there was a downregulation of IBA-1+ microglia at 1 week poststroke.[69] A similar result was observed in another study, in which macrophages were reduced in the brain with low-dose treatments of VEGF.[77] In contrast, higher doses of VEGF increased macrophage density.[77]

In addition to angiogenic growth factors, neurotrophins released by stem cells have been beneficial after transplantation. In a human study involving five stroke patients, BMSCs were harvested autologously from each patient and transplanted at several points at the perilesional area. Neurotrophins, such as BDNF and NGF, were significantly increased in the brain tissue after BMSC transplantation as detected with ELISA.[78] Human MSCs transplanted into rats' brains with ischemic stroke reduced apoptotic cells around the ischemic boundary.[78] In all, trophic factors from transplanted cells have been shown to be protective in stroke brains and attenuate inflammation.

Enhancing trophic factor expression
Genetic upregulation of factors in stem cells

Even though stem cells by themselves are beneficial to the stroke area and the animal as a whole, researchers are pursuing ways to further enhance the beneficial aspects of stem cells. One of the focuses has been to genetically increase their trophic factor release [Table 1]. With the current plethora of plasmid cloning tools available, this approach to trophic factor upregulation has become increasingly feasible. Many plasmid backbones are preconstructed with a promoter, fluorescent labeling tags (e.g., green fluorescent protein), and mammalian and bacterial selection markers. These plasmids are accessible

Table 1: Genetic modifications of stem cells transplanted into stroke models

Factor upregulated	Stem cell type	Upregulation method	Enhanced benefits observed with combination therapy	Citation
BDNF	mMSC	pShuttle2 vector, CMV promoter, adenovirus infection	Reduced motor deficits at 14 days poststroke	[28]
PlGF	hMSC	Fiber-mutant F/RGD adenovirus vector, adenovirus infection	Greater angiogenesis and reduction in lesion volume, and better performance on the limb placement and treadmill stress tests	[79]
Angiopoietin-1	hMSC	pCAcc vector, CAG promoter, adenovirus infection	Enhanced neovascularization and regional cerebral blood flow, and improved performance on the treadmill stress test	[80]
GDNF, CTNF, NT3, BDNF	hMSC	Fiber-mutant adenovirus vector, CA promoter, adenovirus infection	MSC-BDNF and MSC-GDNF resulted in greater reduction of infarct area and better performance on the limb placement test	[81]
Bcl-2	mESC	pcDNA3-based plasmid, CMV promoter, transfected via electroporation	Increased viability and differentiation of transplanted cells and improved evaluation by neurological severity score	[36]

BDNF: Brain-derived neurotrophic factor, PlGF: Placental growth factor, GDNF: Glial cell line-derived neurotrophic factor, CTNF: Ciliary neurotrophic factor, NT-3: Neurotrophin-3, hMSC: Human mesenchymal stem cells, mESC: Mouse embryonic stem cells, mMSC: Mouse mesenchymal stem cells, CMV: Cytomegalovirus, MSC: Mesenchymal stem cells

via global plasmid repositories, such as Addgene, and kits with subcloning instructions. Several groups have shown that the upregulation of trophic factors in transplanted cells can improve stroke outcome even more than unmodified stem cells.[28,36] For example, neonatal rats with ischemic stroke received intranasal delivery of MSCs with upregulated BDNF.[28] In this study, BDNF was upregulated through genetic engineering via adenoviral vector transduction. The BDNF gene was cloned into the pShuttle2 vector under the cytomegalovirus promoter. This plasmid was packaged in an adenovirus. Overall, MSC and MSC-BDNF both showed positive effects on the stroke injury, but some outcomes were more improved with BDNF upregulation in the cells. Even though infarct size and gray matter loss did not show a difference between MSCs versus MSCs + BDNF, some outcomes showed an improvement with BDNF upregulation. Rats transplanted with BDNF-MSCs had an attenuated motor deficit at 4 days after stroke compared to MSCs only.[28] Other growth factors, such as placental growth factor (PlGF), has also been upregulated in MSCs.[79] When transplanted into the brain after stroke, MSCs with PlGF consistently showed a smaller lesion volume measured with magnetic resonance imaging for 24 h to 7 days compared to MSCs without PlGF.[79] Sandwich ELISA assay indicated an *in vivo* production of PlGF after transplantation at 3 and 7 days.[79] Further, a greater reduction in cell death (measured by TUNEL) was observed at the ischemic boundary of animals transplanted with MSCs with PlGF upregulation compared to animals with unmodified MSCs.[79] Finally, G-CSF has already shown great therapeutic potential on its own by enhancing neurogenesis and angiogenesis after stroke and even increasing neuroprotective effects to allow for a greater therapeutic window for tPA administration.[64] However, it has not been well-characterized in combination with stem cell therapy in the context of stroke, although when combined with stem cell factor, another hematopoietic factor, animals displayed improved functional outcomes following MCAO.[82]

Upregulating an anti-apoptotic factor to reduce apoptosis has been shown to be beneficial as well. In our early investigations, ES cells were engineered to overexpressed Bcl-2 which is an anti-apoptosis factor in the intrinsic pathway.[36] Bcl-2-upregulated cells showed better survival than cells without upregulation *in vivo*.[36] Transplantation of the Bcl-2-upregulated cells improved functional recovery in animals with transient cerebral ischemia.[36] Promoting cell transplant survival through the upregulation of survival factors has become increasingly used method in the field of stem cell transplantation for stroke therapy. This approach not only shows protection on transplanted cells themselves but also is beneficial in supporting regeneration and functional recovery.

Hypoxic preconditioning in stem cells

There are other ways to upregulate trophic factors in stem cells. The use of hypoxic preconditioning has been a clever strategy to induce the expression of trophic factors in transplanted stem cells. This strategy is becoming more commonly used as a way to bolster cell survivability and to increase trophic factors. One of the major obstacles of stem cell transplantation into the stroke brain is that a large proportion of the transplanted cells fail to fully differentiate or survive for long period posttransplantation. In one transplantation paradigm, many

of the transplanted cells fail to survive beyond 3 days.[83] Exogenous cell death may be due to a hostile host environment that contains cytotoxic elements, including inflammation and oxidative stress, during the acute phase of ischemic stroke or in part due to mechanical injury from the harvesting of the cells for transplantation and the injection itself. Cell transplantation must be timed so as to minimize the stem cell exposure to cytotoxic substrates and inflammation associated with ischemic cell death. Aside from optimizing the transplantation window, hypoxic preconditioning is another strategy to enhance cell transplantation survival.

Preconditioning is the phenomenon in which delivering a sublethal stimulus to an organism primes it for a greater subsequent insult. In particular, ischemic and hypoxic preconditioning has been used in animals and cell culture to increase the tolerance of organisms and cells to a subsequent insult. In fact, while preconditioning is an effective strategy for improving upon stem cell therapy, it can also apply to an entire organism, such as through sublethal global ischemia, which subsequently promotes endogenous neurogenesis and neuroblast migration.[84] One of the first studies of ischemic preconditioning was reported by Dahl and Balfour in 1964.[85] They showed that exposing a rat to a brief period of anoxia allowed the rat to tolerate and survive a prolonged anoxic exposure.[85]

Ischemic and hypoxic preconditioning primes the system through the oxygen-sensing hypoxia-inducible factor-1 (HIF-1) system. The HIF-1 system is a major regulator of oxygen homeostasis that induces an adaptive response under hypoxic conditions. Under normoxia or normal oxygen conditions, HIF-1α is constitutively transcribed but degraded by prolyl hydroxylase (PHD) so that tissues at normoxia have very low, almost absent levels of HIF-1α. PHDs require iron, 2-oxoglutarate, and oxygen for its substrates for the hydroxylation of HIF-1α protein. Under normoxia, oxygen is available for PHDs to hydroxylate HIF-1α, targeting it for proteasomal degradation by the von Hippel–Lindau (VHL) E3 ubiquitin ligase. VHL binds to hydroxylated HIF-1α for its degradation. Under hypoxic conditions, PHDs do not have sufficient oxygen to hydroxylate the HIF-1α protein, thus preventing proteasomal degradation and allowing HIF-1α to heterodimerize with HIF-1 β. The dimer then translocates into the nucleus, where it will bind to hypoxia response elements to activate the transcription of adaptive genes, including those for VEGF, EPO, BDNF, and glial cell line-derived neurotrophic factor.[59] Hypoxic preconditioning primes cells and organisms by stimulating the HIF-1 system leading to transcription of adaptive genes before the greater injury.

This preconditioning paradigm has been used in *in vitro* models of cell cultures to bolster their survivability for transplantation.[18,58] For example, investigations from our group show that hypoxic preconditioning of neural-differentiating mouse ES cells for 12 h under 0.1% oxygen 24 h before exposing them to serum deprivation resulted in a fewer TUNEL + cells, caspase-3+ cells, and lower lactate dehydrogenase release.[18] In addition to reducing the indicators of cell death, preconditioning also resulted in the upregulation of a cell survival factor, Bcl-2.[18] The priming under 0.1% oxygen hypoxic conditions enhanced the cells' endogenous protective mechanisms to

allow greater tolerance to the subsequent insult of serum deprivation. Similarly, in human ES cells differentiated into neural progenitors, hypoxic preconditioning for 12 h at 0.1% oxygen resulted in upregulation of HIF-1, VEGF, EPO, and Bcl-2.[58] Furthermore, the preconditioned neural progenitors were also more likely to differentiate into neurons as compared to cells that had not been preconditioned.[58] The implications for the implementation of preconditioning before transplantation are great given the multifactorial benefits, including enhancement of neural regenerative potential and the greater release of trophic factors to benefit both the local microenvironment and the surrounding tissue.

BMSCs have also shown a similar response to preconditioning. In a focal ischemic stroke model, intranasally transplanted cells that were hypoxia-preconditioned showed an increase in expression of CXCR4, MMP-2, and MMP-9 after preconditioning.[26] These contributed to the improved outcomes in stroke mice due to the increased migratory behavior of the transplanted cells. The cells also showed better survival with hypoxic preconditioning. Finally, animals receiving preconditioned cells showed greater behavioral recovery at 14 days after stroke compared to those that received cells without preconditioning.[26]

Conclusions

Recent progress in stem cell transplantation, especially with iPS cells, is on the forefront of regenerative research. Compelling evidence endorses that cell therapy, trophic support, and the combination of the two by genetic engineering demonstrate neuroprotective and regenerative effects and increase functional recovery after experimental stroke. In addition, several methods have been investigated to enhance the beneficial effects of stem cells after transplantation, including matrix scaffolds to improve cell viability and differentiation, as well as hypoxic preconditioning to increase trophic factor expression and the HIF-1 pathway activity. Some clinical trials are already underway to study the effects of stem cell transplantation for stroke,[29-31] but there are still many challenges to be optimized in terms of cell type, cell survivability, dosage, and timing before stem cell transplantation can be commonly used for stroke treatments. One of the most important challenges facing cell therapy will be the restoration of lost circuits through cell replacement, and this may be addressed by combining stem cell therapy with other approaches such as optogenetics and physical rehabilitation or other physiological stimuli. Furthermore, as we continue to advance both the fields of stem cell and stroke research and move closer toward the ultimate goal of regenerative therapy, greater ethical questions will arise and gray areas will emerge. As a result, universal guidelines will need to be established for stem cell transplantations for stroke. The Stroke Therapy Academic Industry Roundtable Consortium has already created a series of criteria for evaluation of preclinical studies of stem cell therapy, known as Stem Cell Therapeutics as an Emerging Paradigm for Stroke.[86] Creating a system for objectively and efficiently approaching stem cell therapy for stroke will streamline future translational research and ensure a more positive reception of the therapy once it reaches fruition.

Financial support and sponsorship

Conflicts of interest
There are no conflicts of interest.

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