Chapter 2
Hypoxia-Primed Stem Cell Transplantation in Stroke

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Abstract Spanning the past decade, stem cell research has made rapid progress, and stem cell transplantation in stroke has emerged as a promising treatment. Clinical applications of the cell-based therapy can benefit from the protective mechanisms of ischemic/hypoxic preconditioning. Genetic engineering techniques have been applied to the development of novel stem cell lines and augmenting the differentiation potency of different stem cells, which may ultimately provide far-reaching applications for translational studies, as well as developmental and pathological models.

Keywords Mesenchymal stromal cells · Neural progenitor cells · Pluripotent stem cells · HIF-1α · Hypoxic preconditioning

Spanning the past decade, stem cell research has made rapid progress, and stem cell transplantation in stroke has emerged as a promising treatment. Clinical applications of the cell-based therapy can benefit from the protective mechanisms of ischemic/hypoxic preconditioning. Genetic engineering techniques have been applied to the development of novel stem cell lines and augmenting the differentiation potency of different stem cells, which may ultimately provide far-reaching applications for translational studies, as well as developmental and pathological models.

Hypoxic and ischemic models are widely used in the research and development of new drugs and clinical therapy. Hypoxic and ischemic conditioning induced by a sublethal stimulus is an adaptive effect that confers enhanced

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resistance to subsequent injuries that would have otherwise been lethal. Conditioning treatments on remote tissues or organs (remote conditioning) demonstrate great therapeutic efficacy with high translational potential. Transplantation of hypoxia-preconditioned cells is one of the feasible strategies that incorporate hypoxic tolerance in clinical applications. Hypoxic preconditioning and stem cell therapy display tremendous synergistic benefits in preclinical and clinical studies [1].

Ischemic/hypoxic preconditioning involves many endogenous defense mechanisms to induce cellular tolerance and therapeutic potentials. Preconditioning triggers include hypoxia/anoxia or exposure to agents such as apelin, carbon monoxide, cobalt protoporphyrin, diazoxide, erythropoietin (EPO), hydrogen dioxide (H_2O_2), heat shock protein (Hsp), hydrogen sulfide (H_2S), insulin-like growth factor-1 (IGF-1), isoflurane, lipopolysaccharide, and stromal-derived factor-1 (SDF-1).

After an effective preconditioning strategy, intranasal delivery of stem cells following ischemic stroke can be delivered into the brain within 24 h of stroke onset. Cells are able to reach the ischemic cortex and deposit outside of blood vessels as early as 1.5 h after administration. In hypoxia-primed stem cell transplantation for stroke, the transplanted preconditioned cells have a multitude of superior attributes, including: (a) enhanced survival and migration to replace damaged tissue, (b) suppression of inflammatory cytokines and downregulation of host immune responses against the allograft, (c) increased trophic factors and stimulation of regenerative healing to promote recovery. Notably, transplantation of preconditioned cells improved homing and integration to the lesion site. There is also greater integration of stem cells due to enhanced maturation and differentiation and the potential ability to promote host angiogenesis, arteriogenesis, neurogenesis, and synaptogenesis.

Stem cell and progenitor cell-based therapies using mesenchymal stem/stromal cells (MSC), endothelial progenitor cells (EPC), hematopoietic stem cells (HSC), oligodendrocyte progenitor cells (OPC), pluripotent stem cells (PSC) and c-kit+ cell population have been under extensive pre-clinical and clinical investigations for a variety of disorders.

Pluripotent stem cells PSCs, such as embryonic stem cells (ESC), are able to make cells from all three germ layers and have the potential to generate any cell/tissue for regeneration. Regardless of their cell source, PSC are capable of self-renewal and give rise to multiple specialized cell types in vitro and after transplantation. Human cell lines derived from ESC, as well as the creation of adult-induced pluripotent stem cells (iPSC) that allow for autologous applications for disease treatments, are most promising in stem cell therapy [2].

This chapter mainly discusses the potential applications of ESCs derived from the inner mass of blastocysts, iPSC reprogrammed from somatic cells, and adult MSCs available for intranasal treatment delivery. Generally, preconditioning of these cell types enhances cell adhesion and increased differentiation into vasculature. Hypoxia may promote neural differentiation of the above stem/progenitor

cells. In addition, ESCs and iPSC are being combined with gene-editing techniques, which enable not only enhanced cell replacement, trophic support, drug delivery, immunomodulatory and anti-inflammatory effects, but also checkpoints and quality control functions for many on-going clinical trials. Genome editing greatly expands the understanding of pathological processes by studying cellular/disease models, as well as human cells and tissue, in which the programmable nucleases can directly correct or introduce genetic mutations. Compared to traditional drug therapy, therapeutic genome editing strategies provide an alternative method to treat both genetic diseases and acquired diseases that have genetic associations.

Strokes are devastating disorders that have complex pathophysiology that arise from a primary hypoxic insult. The initial insult causes a dysfunction of energy metabolism followed by massive cell death, glutamate excitotoxicity, free radical damage, reactive gliosis, activation of apoptotic cascades, acute and chronic inflammation, and other pathological pathways [3]. Hypoxia preconditioning applied to stem cells have been shown to enhance resistance to those injurious insults. The hypoxia-primed stem cells can increase pro-survival and anti-inflammatory signals, hypoxia-inducible factor (HIF)-1, trophic/growth factors, protein kinase B (Akt), extracellular signal-regulated kinase, glycogen synthase kinase-3β, matrix metalloproteinase-2, survivin, and B-cell lymphoma 2 (Bcl-2). HIF-1α and HIF-1β are nuclear factors with central roles in hypoxic/ischemic preconditioning and neuroprotection against ischemic injury. As a low-oxygen sensor, its translocation and activation in the nucleus results in production of several downstream genes such as CC chemokine receptor-7 (CCR-7), C-X-C chemokine receptor type 4 (CXCR-4), EPO, lactate dehydrogenase A, c-Met, matrix metalloproteinase-9 (MMP-9), pyruvate dehydrogenase kinase-1, sodium-calcium exchanger-1, uncoupling protein-2, and vascular endothelial growth factor (VEGF). HIF-1α stabilization also induces activation of protein kinase C (PKC) through mitochondrial mechanisms. PKC activates nuclear factor-kappa B (NF-κB) signaling, further enhances antioxidant and anti-apoptotic genes such as manganese superoxide dismutase (MnSOD) and Bcl-2, and promotes secretion of brain-derived neurotrophic factor (BDNF), FGF, and VEGF [4].

In adult animals after fetal tracheal occlusion, the blood pressure drops to an extremely low level within several minutes, triggering severe physiological responses. During these conditions, respiratory rate and cardiovascular activity enter an adaptive state. In many moderate to severe ischemic events, oxygen balance and collateral blood flow are controlled to be compensatory responses and the protective mechanisms are activated. Preconditioning harnesses these physiological responses in order to artificially provide equal and sometimes greater benefits than these organic controls. Sublethal ischemic events enhance tolerance to lethal ischemia in the brain and other organs. After preconditioning, neuroplasticity and hypoxic/ischemic adaptation can be induced, which involves changes in physiology, neurochemical, and neuroelectrophysiological properties [5].

2.1 Stem Cells for Intranasal Cell Therapy

Some recent improvements include: (a) *Transplantation of lineage-committed, fully-differentiated cells*. Generation of pure and differentiated specific type of cells are still needed. To eliminate the risk of tumorigenesis, quality control has been applied to the genetic properties of transplanted cells. Suicidal gene knock-in cells are ready for the elimination of potentially tumorigenic cells. (b) *Development for high-quality stable cell lines.* Standard operating procedures are developed to detect genetically-unstable cells and cancer stem cells. (c) *Continuous control of transplanted cells*. Technological advances and increasing corroboration from human studies allow the continuous monitoring of the exogenous cells and preventing tumor formation. (d) *Reducing transplant rejection*. The concern is that the cells after transplantation might trigger delayed transplant rejection. This concern is partially resolved now by utilizing autologous MSCs from the host's bone marrow, adipose tissue, and others, and differentiating iPSC reprogrammed from the host's own somatic cells. Alternatively, MSCs are low immunogenicity cells and show immunosuppressive effects after transplantation.

Ischemia/hypoxia and reactive oxygen species (ROS) are common players in tumorigenesis, stemness of cancer stem cells, and tumor progression [6]. The niche of cancer stem cells identified within many types of tumors or hematological cancers demonstrates hypoxic environment and low ROS conditions. Enforced tumoral expression of CD24, CD133, erythropoietin (EPO), HIF-1/MMPs, Janus kinase (JAK)/signal transducer and activator of transcription (STAT), Kruppel-like factor 4 (KLF-4), NANOG, nestin, Notch signaling, octamer-binding transcription factor 4 (OCT-4), SNAIL1, sodium calcium exchangers, transforming growth factor-1 (TGF-1), mothers against decapentaplegic homolog-4 (SMAD-4), and VEGF are found under the hypoxic lower-ROS conditions. HIF-1 and VEGF are two antitumoral angiogenic targets (e.g. anthracycline chemotherapy), which facilitate mobilization of circulating progenitors to the tumor angiogenesis. Hypoxia also induces stemness via reprogramming. The involved molecules in MSCs are fibroblast growth factor-2 (FGF-2), miRNA-302, NANOG, Notch-1, and OCT-4.

Stem cells provide developmental and pathological models. Based on cell potency and cell types, stem cells are classified as totipotent cells, naïve pluripotent stem cells, primed pluripotent cells, and tissue-specific multipotent stem cells. (a) *Totipotent cells.* Characteristic of the zygote, early blastomeres, and further reprogrammed/extended PSCs develop into all tissues, including extra-embryonic tissue. (b) *Naïve stem cells*. In a ground state, they harbor the prerequisite potential to differentiate into all embryonic lineages and develop into chimeric blastocysts. They possess high clonogenicity and do not carry specification markers. Naïve stem cells display greater levels of pluripotency marker proteins, including OCT-4, NANOG, SOX-2, KLF-2, and KLF-4. (c) *Primed pluripotent cells. e.g. human ESC.* They do not produce chimeras, express an FGF-5 specification marker and have low clonogenicity. Primed pluripotent cells lose KLF-2 and KLF-4 expression. (d) *Tissue-*

specific multipotent stem cells. They have the least differentiating potency among all stem cell types, with the ability to form tissue-specific cell types.

Cell sources in stem cell therapy may include: (a) *ESC.* They are a useful tool for exploring early embryonic development, modeling pathological processes of diseases, and developing therapeutics through drug discovery and potential regenerative medical treatments. ESC have very high differentiation efficiency into various transplantable progenitors/precursors and terminally differentiated neuronal and glial cell types, including cortical glutamatergic, striatal γ-aminobutyric acid gamma aminobutyric acid (GABA)-ergic, forebrain cholinergic, midbrain dopaminergic, serotonergic, and spinal motor neurons, as well as astrocytes and oligodendrocytes. (b) *iPSC*. They are pluripotent cells that are artificially de-differentiated from adult somatic cells by several transcription factors or small-molecule compounds, which are obtained from patients, and then banked and stored. They harbor much less ethical concern and opposition, and they minimize the risk of immune system rejection [7]. They are amenable as donor cells for cell replacement therapy, disease modeling, and drug screening. (c) *MSC.* They are from the bone marrow, adipose tissue and some other tissues. In stroke therapy, they show immunomodulatory and anti-inflammatory effects. Clinical trials demonstrate safety and feasibility of MSC transplantation in acute and chronic stroke with no tumorigenicity reported following cellular transplantation [8, 9]. (d) *Fetal NPC.* They differentiate into functional neurons and multiple types of neuroglia, allowing for the identification of specific neurodevelopmental processes related to the pathophysiology of developmental disorders. Fetal hNPC were investigated in clinical trials for the treatment of spinal cord injury (SCI) and age-related macular degeneration (AMD), but the safety has not yet been verified.

2.2 Conditioning Medicine and Cell Survival Mechanisms

Hypoxia, ischemia, or limited oxygen levels in different parts of the body all induce systemic changes under some microenvironmental conditions. For example, individuals in mountainous regions show adaptation to a lower oxygen level to maintain normal physiological functions for plateau residents at higher elevations. Neuronal activities consume a large amount of oxygen and glucose for maintenance of normal brain activities. Ischemic/hypoxic insults are more likely to have a greater deleterious effect within the brain. They are also more likely to cause a greater extent of damage in adult brains, as compared with embryos and newborns. In the body, the bone marrow cells survive well in the physiologically hypoxic conditions (1–6% O_2 in the bone marrow) and are potentially homing to the ischemic/hypoxic regions.

HIF-1 is a critical mediator in ischemia/hypoxia and ROS-induced responses. Under hypoxia, HIF-1 is involved in the activation of cytokines/chemokines, transcription factors and microRNAs (e.g. miRNAs-34a, 210, 214) for cell survival,

metabolic adaptation, mitophagy, and mitochondrial biogenesis, and regulation of neurotrophic/angiogenic factors. HIF-1 isoforms upregulate β-catenin transcription and activate multiple proteins, including the activator protein 1, aryl hydrocarbon receptor (AhR), AhR nuclear translocator, bone morphogenetic protein (Bmp), cAMP response element-binding protein (CREB), cystathionine γ-lyase, cAMP-1-activated exchange protein Epac-1, forkhead box O3 (Foxo3), and elevation in hypoxia response element, MMPs, and sex-determining region Y box (SOX)-1. They also increase the expression of glucose-6-phosphate transporter, glucose transporter 1/3. HIF isoforms are coactivator of cell growth and autophagy regulator mechanistic target of rapamycin (mTOR), parkinsonism associated deglycase PARK7, multifunctional protein pyruvate kinase isozymes M1/M2 (PKM1/2).

2.3 Mitochondrial Mechanisms in Stem Cell and Stroke Treatment

Preconditioning results in better survival of bone marrow mesenchymal stromal cells and neural progenitor cells in vitro and/or after transplantation. This is particularly relevant for cell therapy because the survivability of transplanted cells is the primary issue after the cells are transplanted into the ischemic brain. Mitochondrial adaptation is one of the important protective mechanisms in preconditioned stem cells and stroke treatment [10, 11].

Mitochondrial ROS production triggered by H_2O_2, H_2S, and/or CO in ischemic brains and transplanted MSCs can induce ischemic/hypoxic tolerance mechanisms. Heme oxygenase-1 inducer cobalt protoporphyrin IX (CoPP) induces generation of endogenous CO and increases H_2O_2 to trigger the tolerance. Other antioxidant gene mediators include COX-2, Nrf2, and stanniocalcin-1. These protein/enzymes in the transplanted cells, as well as in the surrounding tissue, can retain survival signals, maintain cellular ion homeostasis, and regulate the balance between oxidative stress and glycolytic metabolism in mitochondria.

Decreased energy demands prevail under hypoxic and ischemic conditions as a compensatory response. However, severe ischemia-induced massive neuronal cell death, and endothelial/extracellular matrix (ECM) damage causes disruption of the blood-brain barrier. Ischemia/hypoxia-upregulated heat shock proteins (Hsp) including Hsp70 and Hsp90 can inhibit the mitochondrial release of second mitochondria-derived activator of caspase and prevent activation of caspase-9 and caspase-3. Hsp90 and Hsp70 may form a complex with Cx43 and facilitate the translocase of the outer membrane 20 (TOM20)-mediated translocation of Cx43 onto inner mitochondrial membranes. Hypoxic conditioning-induced HIF-1α stabilization reduces oxidative phosphorylation, leads to the opening of mitochondrial K_{ATP} channels and activates PKC.

2.4 Post-ischemic Flow Recovery

Reduction of blood oxygen and glucose levels, or an ischemic event caused by occlusion of blood vessels in the brain impact neuronal cell survival and neural function. Conditioning shows great benefits of neuroprotection and local cerebral blood flow recovery. Manipulation of hypoxic preconditioning, perconditioning, and postconditioning within the sublethal range in physiological and pathological conditions all show priming effects of improving the tolerance of cells, tissues, and the whole body from recent, on-going, and future insults.

Paracrine release of VEGF and EPO stimulate endogenous arteriogenesis, angiogenesis, and neurogenesis after ischemic stroke. HO-1/CO and SIRT1/eNOS/NO that regulate the cerebral vasodilation may also contribute to collateral circulation and flow recovery. Many genes of angiogenesis and arteriogenesis may be involved in the stem cell benefits and post-ischemic flow recovery (Table 2.1).

Hypoxic conditions induce the mobilization of endogenous stem cells. An ischemic insult to the cortex markedly increases SDF-1 in the ischemic region, a chemoattractant for directional migration of neuroblasts expressing CXCR-4. Hypoxia can also induce migration in various types of cells, including BMSC, cardiac SCA-1+ progenitors, ESCs/iPSC, NSC, and some tumor cells. The SDF-1/CXCR-4 axis and hypoxia are mediators for MSCs/EPC migration in the bone marrow, the peripheral blood, and many other organs. Activated SDF-1/CXCR-4 axis and monocyte chemoattractant protein 1 (MCP-1)/C-C chemokine receptor type 2 (CCR2) play important roles in migration of NPC and EPC after ischemic stroke, which direct the migrating neuroblasts to the infarct region for regeneration of the neurovascular network. Similarly, as treatment for myocardial infarction, hypoxia-induced upregulation of CXCR-4 in CD34+ stem/progenitor cells facilitated recruitment of donor CD34+ cells to the heart to protect against ischemia-reperfusion injury.

Mobilization of stem cells from the bone marrow demonstrates great therapeutic potentials. Bmp, EPO, granulocyte colony stimulating factor (G-CSF), and interleukin-10 (IL-10) mobilize endogenous bone marrow cells from the bone marrow, increase the homing and differentiation of NSC (originated from the neurogenic niches within the brain) and MSCs into the peri-infarct regions, and exert neuroprotective effects to promote stroke recovery and mitigate stroke damage. G-CSF mobilizes CD34+ hematopoietic stem cells and reduces microglial activation. Fasudil, an inhibitor of Rho kinase, is used to increase the G-CSF level for mobilization. In chronic hypoxia secondary to pulmonary hypertension, when migratory adaptation to SDF-1 and cell adhesion are significantly inhibited, hypoxic EPCs with both upregulated VEGFR-2+ and CXCR-4+ are insufficient for vascular remodeling. Enhancement of EPO/EPOR is demonstrated to attenuate hypoxia-induced pulmonary hypertension, while EPOR (−/−) mice display failed mobilization of EPCs to pulmonary endothelium for repair [12]. MMPs and natural MMP inhibitors are involved in ECM stabilization, glial activation, and regulation of migratory factors for stem cells and some other cells.

Table 2.1 Expression and mechanism of angiogenesis and arteriogenesis genes in stem cells

Gene family	Gene	UniProtKB ID	Cell type	Tissue source	Related cell	Functions
Peptidase M2 type	Ace	P12821	HSC	BM	–	Vascular morphogenesis
G-protein coupled receptor	Ackr3	P25106	NSC	SVZ	Microglia	Cell migration, chemotaxis
			MSC	BM		
	Adgrg1	Q9Y653	HSC	BM	–	VEGF-A production
	Ccr2	P41597	HSC	BM	Monocyte	Cell migration, adhesion
			NSC	SVZ		
	Cxcr3	P49682	NSC	SVZ, DG	–	Endothelial cell proliferation, survival, and chemotaxis
Hepatokine	Angptl3	Q9Y5C1	HSC	BM	–	Endothelial cell adhesion, and migration
Ankyrin SOCS box family	Asb5	Q8WWX0	–	–	Myogenic progenitor	Arteriogenesis
ATPase α/β chains family	Atp5f1b	P06576	–	–	–	Endothelial cell migration
–	Bcas3	Q9H6U6	–	–	–	Endothelial cell migration
TGF-β family	Bmp4	P12644	MSC	BM	Astroglia	Endothelial tube morphogenesis, endothelial cell proliferation, differentiation, migration
			NSC	SVZ, DG		
	Nodal	Q96S42	–	–	Macrophage	Angiogenesis
	Tgfb2	P61812	NPC	–	Neuron	Endothelial cell capillary morphogenesis, vascular smooth muscle cell proliferation, and migration
–	Bmper	Q8N8U9	HSC	BM	–	Endothelial cell activation, proliferation, migration
Glycosyltransferase family	B4galt1, C1galt1	P15291, Q9NS00	–	–	–	Cell adhesion, chemotaxis, cell survival

Collagen calcium-binding EGF domain-containing	Ccbe1	Q6UXH8	–	–	–	Endothelial cell migration
CD34 family	Cd34	P28906	HSC, MSC	BM	–	Endothelial cell proliferation, and adhesion
CMGC Ser/Thr PTK	Cdk5	Q00535	NSC	DG	Microglia	Endothelial cell migration, lamellipodia formation
	Mapk14	Q16539	NSC	SVZ, DG	Microglia	Angiogenesis
Carcinoembryonic antigen	Ceacam1	P13688	NPC	–	–	Endothelial cell differentiation, migration, sprouting angiogenesis, Arteriogenesis
β-Catenin family	Ctnnb1	P35222	NSC	SVZ, DG	Microglia	Endothelial tube morphogenesis, cell differentiation
			MSC, HSC	BM		
DimethylargininaseFamily	Ddah1	O94760	NPC	–	–	Regulation of arterial blood pressure
–	Dll1	O00548	NPC	–	Myogenic progenitor	Sprouting angiogenesis, cell migration
			HSC	BM		
RTK	Epha1, Epha2, Epha4, Ephb1, Ephb2, Ephb3, Ephb4	P21709, P29317, P54764, P54762, P29323, P54753, P54760	NSC	DG	Myogenic progenitor, neuron	Sprouting angiogenesis, endothelial cell proliferation, adhesion
Eph receptor interacting protein	Efna1, Efnb2, Efna3	P20827, P52799, P52797	–	–		
RTK	Flt1/VEGFR1	P17948	HSC	BM	Macrophage	Sprouting angiogenesis, cell survival, migration, and chemotaxis

(continued)

Table 2.1 (continued)

Gene family	Gene	UniProtKB ID	Cell type	Tissue source	Related cell	Functions
Heparin binding growth factor	Fgf2, Fgf10	P09038, O15520	iPSC	–	VSMC	Endothelial cell proliferation, chemotaxis, sprouting angiogenesis
			HSC	BM		
Tyr PTK	Fgfr1, Fgfr2	P11362, P21802	NSC	OB, SVZ, DG	Neuron	Endothelial cell proliferation, migration, Chemotaxis
	Tie1, Tek/Tie2	P35590, Q02763	EPC	Blood		Sprouting angiogenesis; promote vascular stability
Forkhead box TF	Foxc1	Q12948	HSC, MSC	BM	–	Endothelial cell migration
	Foxo1, Foxo4, Foxp1	Q12778, P98177, Q9H334	NSC	Embryo		Endothelial cell proliferation, tube formation, migration
–	Gata2	P23769	HSC	Blood	Neuron	Endothelial cell proliferation
GRB2/Sem-5/DRK family	Grb2	P62993	NPC	–	–	Endothelial cell migration
Histone deacetylase family	Hdac5	Q9UQL6	NSC	DG	Neuron	Sprouting angiogenesis, cell migration
High mobility group proteins	Hmga2	P52926	HSC	BM	Myogenic progenitor	Cell proliferation
			NSC	SVZ		
Antennapedia homeobox	Hoxa7	P31268	MSC, HSC	BM	–	Cell differentiation
bHLH TF	Id3	Q02535	NSC	SVZ, DG	Astroglia	Gliovascular coupling
	Myc	P01106	iPSC	–	–	Sprouting angiogenesis
	Tal1	P17542	HSC	BM	–	Form microvasculature
–	Jag1	P78504	NSC	SVZ, DG	–	Sprouting angiogenesis
Jumonji-C domain containing protein	Jmjd6	Q6NYC1	MSC	Adipose	–	Sprouting angiogenesis

TCF/LEF family	Lef1	Q9UJU2	MSC	Adipose, BM	–	Sprouting angiogenesis
			NSC	DG		
Lysyl oxidase	Loxl2	Q9Y4K0	NPC	–	–	Endothelial cell proliferation, migration, sprouting angiogenesis
–	Naa15	Q9BXJ9	–	–	–	Regulation of vascular permeability
Basic leucine zipper TF	Atf2, Nfe2l2	P15336, Q16236	HSC	BM	Microglia, astroglia	Endothelial cell migration, survival
			NSC	SVZ		
Type-1 TM	Notch1	P46531	NSC	SVZ, DG	Microglia, astroglia, myogenic progenitor	Endothelial cell fate commitment, cell differentiation, gliovascular coupling
			MSC, HSC	BM		
CCN	Nov/Ccn3	P48745	HSC	BM	VSMC, neuron	Cell adhesion, chemotaxis, cell survival
Nuclear hormone receptor	Nr2e1	Q9Y466	NSC	SVZ, DG	Microglia, astroglia	Cell cycle progression
Plexin family	Plxdc1	Q8IUK5	–	–	–	Endothelial cell capillary morphogenesis
PI3K/PI4K	Pik3ca	P42336	–	–	Neuron	Endothelial cell migration, vasculogenesis
TKL Ser/Thr PTK	Raf1	P04049	HSC	BM	Macrophage, neuron	Cell motility, proliferation
Su(H) family	Rbpj	Q06330	–	–	Astroglia	Endothelial cell fate commitment and specification
–	Runx1, Runx2	Q01196, Q13950	NSC	SVZ, DG	Astroglia	Endothelial cell proliferation
			MSC	BM		

(continued)

Table 2.1 (continued)

Gene family	Gene	UniProtKB ID	Cell type	Tissue source	Related cell	Functions
–	Serpine1	P05121	MSC	BM	Astroglia	Endothelial cell survival
Histone lysine methyltransferase	Setd2	Q9BYW2	NSC	–	Myogenic progenitor	Vascular remodeling
			HSC	BM		
Secreted frizzled-related protein	Sfrp2	Q96HF1	HSC	BM	Neuron	Hematopoietic stem cell proliferation
			NSC	–		
–	Shb	Q15464	HSC	BM	Neuron	Endothelial cell proliferation, and differentiation
–	Shc1	P29353	NSC	SVZ	–	Endothelial cell migration, sprouting angiogenesis
Hedgehog	Shh	Q15465	NSC	SVZ, DG	Microglia, astroglia	Chemotaxis, vasculogenesis
Sirtuin protein deacetylases	Sirt1	Q96EB6	NSC	DG	Microglia, astroglia	Endothelial cell migration, proliferation
Dwarfin/SMAD family	Smad1	Q15797	NSC	SVZ	Neuron, myogenic progenitor	Endothelial cell proliferation, sprouting angiogenesis
			MSC, HSC	BM		
–	Srf	P11831	HSC	BM	Neuron, myogenic progenitor	Sprouting angiogenesis, cell migration
STAT TF	Stat3	P40763	NSC	SVZ, DG	Neuron, myogenic progenitor	Endothelial cell proliferation
			MSC	BM		
Sry-type HMG box	Sox17	Q9H6I2	NSC	–	–	Endothelial cell differentiation, vascular morphogenesis
	Sox18	P35713	MSC	BM		Endothelial cell migration, establish blood-brain barrier
RTase family	Tert	O14746	NSC	DG	–	Endothelial cell survival

Class I aminoacyl tRNA synthetase	Wars	P23381	–	–	–	Shear stress
Wingless/integrated	Wnt5a, Wnt7a, Wnt7b	P41221, O00755, P56706	HSC	BM	Neuron	Endothelial cell proliferation, migration, neurovascular coupling
			NSC	SVZ, DG, OB		
CCCH-type ZFP	Zc3h12a	Q5D1E8	NPC	–	Macrophage	Endothelial cell differentiation, gliogenesis
			MSC	UC, BM		

BM bone marrow, *DG* dentate gyrus, *EPC* endothelial progenitor cell, *HSC* hematopoietic stem cell, *iPSC* induced pluripotent stem cell, *MSC* mesenchymal stromal cell, *NPC* neural progenitor cell, *NSC* neural stem cell, *SVZ* subventricular zone, *TF* transcription factor, *TM* transmembrane, *OB* olfactory bulb, *PTK* protein kinase, *RTK* receptor tyrosine protein kinase, *UC* umbilical cord, *ZFP* zinc finger protein

Preconditioning with growth factors including FGF-2, glial cell line-derived neurotrophic factor (GDNF), IGF-1, SDF-1, and transforming growth factor-alpha (TGF-α), enhances paracrine effects. Upregulated factors may include angiopoietin-1, BDNF, EPO, FGF-2, GDNF, hepatocyte growth factor, MMP-2, placental growth factor, SDF-1, and VEGF. Treatment effects include: reduction of neurotoxicity and cell apoptosis, promotion of angiogenesis, neurogenesis, and synaptogenesis, and attenuation of functional and pathophysiological decline. Secreted factors also benefit the engraftment of stem/progenitor cells by enhancing cell survival, differentiation, and integration.

Enhancement of migration and homing of transplanted cells are relevant to the efficacy of cell-based therapy. Homing and promoted paracrine activity of endogenous stem cells as well as transplanted cells greatly contribute to flow recovery [13]. Plasma levels of SDF-1α and VEGF significantly increase during the subacute phases of ischemic stroke. Increased VEGF and SDF-1 in peripheral blood are involved in recruitment of EPC.

SDF-1/CXCR-4/CXCR-7 axis are well-known players in many cellular, physiological, and pathological processes, such as cell proliferation, migration, chemotaxis, inflammation, neurogenesis, angiogenesis, and hematopoiesis. In the peri-infarct regions of ischemic stroke, SDF-1 increases and interacts with CXCR-4/CXCR-7+ cells, leading to neurogenesis and neurovascular repairs. Upregulation of CXCR-4 and hepatocyte growth factor receptor, and activation of extracellular signal-regulated kinase (ERK), PI3 kinase-AKT, or PLCγ-PKC, MMP-2, and MMP-9 in MSCs by preconditioning insults enhances the migration and homing.

Circulating VEGF, TNF-α, and IL-8 also impact the recruitment of c-Kit+/Tie-2+ EPCs, CD34+ HSCs and MSCs towards the infarcted area. VEGF-A is an apoptosis inhibitor of vascular smooth muscle cells controlled by TGF-β/SMAD-3 signaling [14]. TGF-β signaling and TGF-β family genes such as Bmp4, Nodal, and Tgfb2 promote proliferation of endothelial cells and vascular smooth muscle cells for angiogenesis and arteriogenesis. VEGFR-1/Flt1 and VEGFR-2/Flk1 are essential for the mobilization and sprouting angiogenesis. Many chemokine and angiogenic genes are upregulated after hypoxic induction on bone marrow-derived hemangioblasts, which promote differentiation toward endothelial lineage and promote neovascularization. These include: Sonic Hedgehog (SHH), miRNA-31, miRNA-132 and miRNA-720 may be involved in EPC-mediated angiogenesis and neovascularization induced by angiopoietin-1, Eph family receptor-interacting protein B2 (ephrin B2), HIF-1α, methyl-CpG-binding protein 2, Ras-GTPase-activating protein, SDF-1, and VEGF. Angiotensin II pretreatment activated the AT1R/HIF-1α/ACE axis in rat BMSC and promoted VEGF production and the angiogenic response. Some negative regulators include: adhesion G protein-coupled receptors, angiopoietin-2/4, miRNA-377, Krueppel-like factor 4, leukemia inhibitory factor, Rho-associated protein kinases, semaphorins, and many others.

2.5 Stem Cells and Neuroplasticity

During the long-term period following ischemic stroke, there are neurodegenerative processes with gradual losses of neurons and neural connections. These include the interactions between neurons, astrocytes, microglial cells, oligodendrocytes, and stem/progenitor cells. To achieve the goal for cell-based repairs, cell type-specific commitment and differentiation protocols are developed [15].

Enhanced migration of endogenous NPC from the lateral ventricle subventricular zone (SVZ) towards the lesion sites increased the potential for cortical regeneration and repair. Wnt signaling players such as Wnt-3a, Wnt-5a, and Wnt-7a/b promote the neuroblast migration and differentiation contributing to neurogenesis, angiogenesis and neurovascular coupling. For example, intranasal delivery of Wnt-3a to the ischemic brain shows neuroprotection and greatly enhances neurogenesis, angiogenesis, and flow recovery [16, 17]. Another related factor in hypoxia preconditioned MSCs is Wnt-4, which modulates axonal growth. NSC and NPC in the adult brain proliferate and migrate from the two regenerative niches, the forebrain SVZ and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. Some other molecules that are shown to promote angiogenesis and arteriogenesis may also activate neural stem cells and neural progenitor cells stimulate regenerative processes such as endogenous neurogenesis. These genes include but are not limited to: Ackr3, Atf2, Bmp4, Ccr2, Ctnnb1, Mapk14, Nfe2l2, Fgfr1, Fgfr2, Hmga2, Id3, Jag1, Notch1, Nr2e1, Runx1, Runx2, Shc1, Shh, Smad1, and Stat3.

Regenerative neuronal circuits after stroke in rodent models include: (a) the interneurons originating from migrating neuroblasts along the SVZ and rostral migratory stream, toward the peri-infarct regions, striatum and the olfactory bulb; (b) the SGZ neuroblasts differentiate and integrate within the hippocampus. Lipid accumulation, perturbation of the microenvironmental fatty acid metabolism, and inhibition of Wnt signaling and VEGFR signaling, have been shown to suppress the homeostatic and regenerative functions of NSC and NPC. Inhibitor of CXCR-4 such as Plerixafor (also known as age-related macular degeneration, AMD-3100) significantly increases VEGFR-2-positive cells in the peripheral blood, elevates SDF-1 levels, and promotes blood vessel formation in an ischemic flap model. Co-culture of neurons with SDF-1-secreting olfactory ensheathing cells after oxygen-glucose deprivation (OGD) treatment, an in vitro method of hypoxic/ischemic preconditioning, showed enhanced neurite outgrowth. In addition, SDF-1-overexpressed NPC derived from iPSC show enhanced axonal and synaptic growth, and increased numbers of NeuN/BrdU and Glut-1/BrdU co-labeled cells in peri-infarct regions after transplantation.

Hypoxia affects the NSC phenotype, cell differentiation, and regenerative repair activity. The proliferation and self-renewal of NSC are maintained under hypoxic conditions. HIF-1α overexpression promotes NSC proliferation and differentiation after intracerebral hemorrhage and hypoxic/ischemic injury. Other related factors include: angiotensin II (Ang II), angiotensin-converting enzyme (ACE), Ang II receptor Type 1 (AT1R), EPO/EPOR, Leukemia inhibitory factor (LIF)/pSTAT-3,

LIF receptor (LIFR)/glycoprotein (gp130), Neurogenin-1/BMP-4, Notch-1, atypical protein kinase C/CREB-binding protein pathway, SOX-2, and VEGF/VEGFR. HIF-1α is also required for neural stem cell maintenance in the adult mouse SVZ as well as for mouse embryonic stem cells toward a neural lineage.

Glial progenitor cells in the SVZ and the white matter proliferate and differentiate after ischemia, which is further augmented by exogenous treatments, including epidermal growth factor (EGF), EPO, GDNF, memantine, and uridine diphosphoglucose glucose, and by the enhancement of BDNF, SHH, VEGF, and Wnt signaling in the SVZ or in transplanted cells. MSCs may produce some other factors including Ang-2, FGF, GDF-5, HGF, IGF-1, LIF, MCP-1, SCF, TGF-β, TIMP-1, TIMP-2, and TSP-1 [18].

Enhanced neuronal maturation, oligogenesis, and synaptogenesis by preconditioning is an effective approach that is logically related to the regenerative efficacy of stem cell-based therapies. Hypoxic preconditioning increases secretion of growth factors and upregulation of their cognate receptors, such as CXCR-4, as well as greater expression of neuregulin-1 isotype β-1/ErbB4, neurofilament, stem cell antigen 1, and synaptophysin. Neuregulin-1 isotype β-1/ErbB4 signaling protects OPCs during and after a hypoxic event in the white matter.

2.6 Inflammation, Immune Responses, and Regeneration

The increased inducible nitric oxide synthase (iNOS), an antioxidant genes, are involved in the regulation of cell fate in the inflammatory microenvironment. Preconditioned cells show inhibitory effects on cyclooxygenase production, and they reduce inflammation by releasing anti-inflammatory factors. In MSCs, downregulated expression of pro-inflammatory cytokines/chemokines and receptors include CC3, CC5, CC17, CCL4, CXCR3, CXCL10, IL-1β, IL-6, TNF-α, IFN-γ, and OX-42. Hypoxia-preconditioned MSCs suppress microglial activation and gliosis in the ischemic brain. MSCs inhibit T-cells and natural killer (NK) cells, and they reduce immune responses by decreasing proliferation of immunocytes. Activation of the transplanted cells may further suppress inflammatory and immune responses in host tissues.

Intranasal delivery of preconditioned stem cells improves motor recovery and promotes regenerative activities, which contribute to greater improvement in coordination skills, neuropsychiatric, and cognitive functions. In the investigations on ischemic stroke models, there are significantly more NeuN-positive and NeuN/BrdU-colabeled neurons, MBP myelination, and Glut1-positive and Glut1/BrdU-colabeled cells in the ischemic core and peri-infarct regions. Co-transplanting stem cells may promote revascularization via paracrine effects. Advanced methods are bringing new approaches for enhanced cell quality/adaptability and improved transplantation therapy for human diseases.

Stem cell transplantation after ischemic stroke stimulates angiogenesis, ameliorates ischemia-hypoxia, and provides nutrient support. Intranasal delivery of BMSC

in the acute phase exerts neuroprotective benefits after ischemia. Hypoxic-preconditioned BMSC and neural progenitors showed significant increases in the survival of transplanted cells, homing to the lesion sites, neuronal differentiation, and functional benefits after stroke. Hypoxia-treated hMSC contain a secretive enrichment of trophic factors that provide a suitable preconditioning strategy for enhanced differentiation of endogenous NPC after transplantation.

Disease modeling and drug screening studies using iPSC allow for greater experimental interrogation and convenience compared to transgenic animal models [19, 20]. iPSC provide for the potential use of reprogrammed somatic cells from the patient to establish disease-relevant phenotypes in vitro and to simulate and recapitulate the molecular signatures of pathogenesis during an early stage [21]. A combination therapy with iPSC and genome editing is proposed as a new therapeutic paradigm to introduce protective mutations, to correct deleterious mutations, to eliminate the antigenic/immunogenic signals in the iPSC, or to destroy foreign viral DNAs in the human body. Genome editing of iPSC uses programmable nucleases including (a) *transcription activator-like effector nucleases* (TALENs). In the absence of exogenous template DNA, the programmable nucleases create a double strand break (DSB) in desired regions, but due to the error-prone no homologous end joining (NHEJ) mechanism of re-ligation, an insertion/deletion (indel) mutation is frequently created at the DSB site. (b) *Clustered regularly interspaced short palindromic repeats* (CRISPR)/*Cas9* technology. To ablate the triple repeats, a pair of single-guide RNAs was applied to target both sides during the expansion. Other than NHEJ, the high-fidelity homology-directed repair (HDR)-based mechanism of genome editing is studied to treat deleterious loss-of-function mutations. HDR-based genome editing provides an exogenous repair template of a single-stranded oligodeoxynucleotide and a donor plasmid to correct a mutated allele to be wild type. It could also integrate therapeutic transgenes into a genomic safe harbor site.

Modified iPSC and its derived multiple cell types will be another useful cell source for treating stroke and many other neurological disorders. Here are unmet translational gaps that warrant further investigation: (a) *Identification of MSC derived from iPSC.* (b) *Intranasal delivery of hypoxia-preconditioned MSC derived from iPSC.* (c) *Angiogenesis and arteriogenesis after transplantation.* (d) *Blood flow recovery.* (e) *Neuroplasticity after transplantation.*

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