Distribution Agreement

In presenting this thesis or dissertation as a partial fulfillment of the requirements for an advanced degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis or dissertation in whole or in part in all forms of media, now or hereafter known, including display on the world wide web. I understand that I may select some access restrictions as part of the online submission of this thesis or dissertation. I retain all ownership rights to the copyright of the thesis or dissertation. I also retain the right to use in future works (such as articles or books) all or part of this thesis or dissertation.

Signature:

_____ _____

Monica Chau Date

Induced Pluripotent Stem (iPS) Cell Transplantation as a Treatment for Ischemic Stroke

By

Monica Chau
B.S.

Graduate Division of Biological and Biomedical Science
Neuroscience Program

_____
Ling Wei, MD
Advisor

_____
Nicholas Boulis, MD, PhD
Committee Member

_____
Donald Stein, PhD
Committee Member

_____
Young Sup Yoon, MD, PhD
Committee Member

_____
Shan Ping Yu, MD, PhD
Committee Member

Accepted:

_____
Lisa A. Tedesco, PhD
Dean of the James T. Laney School of Graduate Studies

_____
Date

Induced Pluripotent Stem (iPS) Cell Transplantation as a Treatment for Ischemic Stroke

By
Monica Chau
B.S., Neurobiology, Physiology, and Behavior
University of California, Davis 2007

Advisor: Ling Wei, MD

An abstract of
A dissertation submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science (GDBBS)
Neuroscience
2014

ABSTRACT

Induced Pluripotent Stem (iPS) Cell Transplantation as a Treatment for Ischemic Stroke

By Monica Chau

Ischemic stroke is a leading cause of death and long-term disability in the U.S. Despite its prevalence, there is only one FDA-approved treatment for stroke, tPA (tissue plasminogen activator) which is a thrombolytic drug limited to use within 4.5h after stroke. Currently, no treatment exists for the regeneration of damaged brain tissue. The adult brain undergoes endogenous regeneration, however it cannot fully repair the damage caused by stroke. Induced pluripotent stem (iPS) cells are a novel source of stem cells created by genetically reprogramming one's somatic cells into pluripotent cells. Transplanting such patient-specific cells can circumvent host immune rejection as well as circumvent the ethical limitations of obtaining pluripotent cells from human embryos.

This dissertation demonstrates the use of iPS cell transplantation as a therapeutic in two ways: by providing replacement cells to the infarct, and by releasing trophic factors to the injured tissue enhancing the recruitment of regenerative progenitors. We examine the regenerative capabilities of transplanted iPSC-derived neural progenitors (iPSC-NPCs) with and without stromal-cell-derived factor-1α (SDF-1α) upregulation. Our overall goal is to enhance the recruitment of endogenous progenitors to the infarct.

To support the hypothesis of our first aim, we tested the transplantation of iPSC-NPCs in a model of neonatal ischemic stroke and were able to demonstrate an enhanced neurogenesis and angiogenesis in the peri-infarct area and increased sensorimotor functional recovery. In our second aim, to supplement the chemoattractive factor, SDF-1α that is endogenously expressed after stroke, we transplanted iPSC-NPCs that had stable overexpression of SDF-1α. We demonstrated an increased endothelial progenitor cell recruitment for angiogenesis in mice with stroke and SDF-1α iPSC-NPC transplantation. Furthermore, transplanting iPSC-NPCs and SDF-1α iPSC-NPCs can provide more neuronal cells to the injury and increased functional recovery after stroke.

Overall, these data suggest that iPS cells have great potential as a personalized transplantation therapeutic for the regenerative phase of ischemic stroke. Transplanting SDF-1α iPSC-NPCs employs a two-fold strategy to provide more neuronal cells into the ischemic parenchyma and to enhance the recruitment of endothelial and neural progenitor cells for regeneration.

Induced Pluripotent Stem (iPS) Cell Transplantation as a Treatment for Ischemic Stroke

By
Monica Chau
B.S., Neurobiology, Physiology, and Behavior
University of California, Davis 2007

Advisor: Ling Wei, MD

A dissertation submitted to the faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Graduate Division of Biological and Biomedical Science (GDBBS)
Neuroscience
2014

ACKNOWLEDGEMENTS

I would like to express my tremendous gratitude to everyone that has offered me support and guidance during my graduate studies. I would first like to thank my advisor, Dr. Ling Wei who along with Dr. Shan Ping Yu provided with me with invaluable direction and guidance in my graduate training. Thank you for your mentorship and for teaching me the scientific process. You have taught me how to carry out my own experiments, and how to collect data and interpret a meaningful story from my findings. I would also like to thank Dr. Oskar Laur for his subcloning work as well as Dr. Xinping Huang for her work in creating the lentiviruses for this project. Their expertise was essential to this project. Thank you to my committee members, Drs. Nick Boulis, Don Stein, and Young Sup Yoon for their continued guidance, knowledge, and support. Special thanks to Xiaohuan Gu for carrying out the animal surgeries, Mingke Song and Dongdong Chen for collecting electrophysiology data, Todd Deveau for plasmid/virus optimization, and Cody Wenyan Cao for our project collaboration. Thank you to all of my fellow lab members past and present for your discussions, helpful critiques, and technical support. I would like to thank the American Heart Association for providing me a pre-doctoral fellowship to support this work and I would like also extend my thanks to the National Institutes of Health for their support.

Last but not least, I would like to express my heartfelt gratitude to my biggest supporters, my boyfriend, Brandon Miller and best friend, Jenna Carlsson and to all of my friends and family, my dad and mom, Robert and Jannie Chau, my siblings, Jonathan and Anthony Chau, and Dorothy Chow. Without your support, encouragement, and listening ear, I would not have gotten this far.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Tables

Figures

List of Abbreviations

AHA	American Heart Association
AIF	Apoptosis inducing factor
AKT	Protein Kinase B
AMPA	DL--amino-3-hydroxy-5-methylisoxazole-propionic acid
ANOVA	Analysis of variance
AP	Action potentials
APAF-1	Apoptotic Protease Activating Factor 1
ASA	American Stroke Association
BAD	Bcl-2-associated death promoter
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
BMSCs	Bone Mesenchymal Stem Cells
BrdU	5-bromodeoxyuridine
CAD	Caspase activating DNAase
CAG	Chicken β-actin
CAGG	Chicken β-actin coupled with CMV early enhancer
cAMP	Cyclic adenosine monophosphate
CC	Corpus callosum
CCA	Common carotid artery
CLRD	Chronic lower respiratory disease
CMV	Cytomegalovirus
CNS	Central nervous system
CXCR4	Chemokine receptor-4
DBH	Dopamine Beta Hydroxylase
DCX	Doublecortin
DEPC	Diethylpyrocarbonate
DGG	Gamma-D-glutamylglycine
DISC	Death-inducing signaling complex
DMEM	Dulbecco's Modified Eagle Medium
ECA	External carotid artery
ECL	Enhanced chemiluminescence
EF1α	Human elongation factor 1α
EGTA	Ethylene glycol tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPO	Erythropoietin
ERK	Extracellular signal-regulated kinases
ES	Embryonic stem
ES Cell	ES Cell Fetal Bovine Serum
FBS	
ESC-NPCs	Embryonic stem cell-derived neural progenitor cells
ESPS	Excitatory Postsynaptic Potentials
ET-1	Endothelin-1
FADD	Fas associated death domain

FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FGF	Fibroblast growth factor
FOXG1	Forkhead box G1
GALV	Fibbon ape leukgemia virus
G-CSF	Franulocyte-colony stimulating factor
GDNF	glial cell line-derived neurotrophic factor
GFAP	Glial Fibrillary Acidic Protein
GFP	Green fluorescent protein
Glut-1	Glucose transporter 1
GPCR	G-protein coupled receptor
GVHD	Graft-Versus-Host Disease
H&E	Hematoxylin & Eosin
H_2O_2	Hydrogen peroxide
HIF-1α	Hypoxia-inducible factor-1α
HREs	Hypoxia response elements
HRP	Horseradish peroxidase
HUVEC	Human umbilical vein endothelial cells
i.p.	Intraperitoneal
IACUC	Institutional Animal Car and Use Committee
INA	Intranasal administration
IP3	Inositol trisphosphate
iPS	Induced pluripotent stem
iPSC	Induced pluripotent stem cell
IPSC-NPC	iPSC-derived neural progenitor/ induced pluripotent stem cell-derived neural progenitor cell
IRES	Internal Ribosome Entry Site
LDH	Lactate dehydrogenase
LIF	Leukemia inhibitory factor
LMWH	Low-molecular-weight heparin
LTR	Long Terminal Repeat
MAPK	Mitogen-Activated Protein Kinases
MCA	Middle cerebral artery
MCAO	Middle cerebral artery occlusion
MCP-1	Monocytechemotacticprotein-1
MEF	Mouse embryonic fibroblast
MEK	MAPK extracellular signal regulated kinases
MMPs	Matrix metalloproteases
MRI	Magnetic Resonance Imaging
MSCs	Mesenchymal stem cells
MSCV	Murine stem cell virus
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NBT/BCIP	Nitro-blue tetrazolium chloride/ 5-bromo-4-chloro-3'-indolyphosphate p-toluidine
NGF	Nerve growth factor
NIH	National Institutes of Health
NMDA	N-methyl-D-aspartate
NSS	Neurological Severity Score
OGD	Oxygen Glucose Deprivation
p130Cas	Crk-associated substrate
PI3K	Phosphoinositide 3-kinase
P1GF	Placental Growth Factor
PBS	Phosphate buffered saline
PDL	Poly-D-Lysine
PGK	Phosphoglycerate kinase 1
PHD	Prolylhydroxylase
PKA	Protein kinase A
PLC	Phospholipase C
PS	Phospholipidphosphatidylserine
Pyk-2	Proline-rich kinase-2
RA	Retinoic acid
RT-PCR	Reverse transcriptase-polymerase chain reaction
SCF	Stem cell factor
SCIA	Stromal cell-derived inducing activity
SDF-1α	Stromal cell-derived factor-1α
SDS	Sodium Dodecyl Sulfate
sFRP1	Secreted Frizzled-Related Protein 1
SGZ	Subgranular Zone
SNAP-25	Synaptosomal-Associated Protein 25
SV40	Simian virus 40
SVZ	Subventricular zone
TEA	Tetraethylammonium
TNF	Tumor necrosis factor
tPA	Tissue plasminogen activator
TrkA	Tropomyosin related kinase A
TTX	tetrodotoxin
Tuj-1	β3 Tubulin
TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
UBC	Human ubiquitin C promoter
VEGF	Vascular endothelial growth factor
VEGFD	Vascular endothelial growth factor D
VHL	Von Hippel-Lindau
YFP	Yellow fluorescent protein

Chapter I
Ischemic Stroke

A. ISCHEMIC STROKE

1. Background

Stroke is a neurological disorder that results in the loss of blood supply to an area of the brain due to a vessel occlusion or a vessel bursting bursting. There are two primary types of stroke: ischemic stroke when the vessel is occluded, and hemorrhagic when the blood vessel bursts. When a blood vessel is occluded, it deprives the brain region of oxygen, glucose, and other metabolic substrates resulting in damage to the brain parenchyma. This dissertation will focus primarily on ischemic stroke. Cell death in the affected area occurs through a cascade of several events in the acute and delayed stages of stroke. These events include ionic disregulation, glutamate excitotoxicity, and inflammation. As a result, the tissue in the affected area undergoes heterogenous cell death including necrosis and apoptosis.

2. Epidemiology

Stroke is one of the leading causes of death and long-term disability in the United States and worldwide[1]. It currently ranks as the fourth cause of death behind heart disease, cancer, and chronic lower respiratory diseases (CLRD). Of all strokes, 87% are ischemic, 10% are hemorrhagic, and 3% are subarachnoid hemorrhage[1]. Every year, the American Heart Association compiles a report with data from the Centers for Disease Control and Prevention and the National Institutes of Health with the latest statistics on cardiovascular disease in the U.S[1]. Approximately 795,000 individuals a year experience a stroke in the U.S. which can result in death or disability[1]. Of the 795,000 attacks, about 610,000 of them are first-time occurrences and 185,000 are recurrent. On average, a

person has a stroke every 40 seconds in the U.S. and every four minutes, someone in the U.S. dies from stroke. The direct medical costs from stroke in 2009 totaled to $22.8 billion[1]. Stroke is the most prevalent in the southeast area of the U.S. (also known as the "stroke belt"), in older adults, those with low socioeconomic status, and blacks[1,2]. The stroke belt consists of the states of North Carolina, South Carolina, Georgia, Tennessee, Mississippi, Alabama, Louisiana, and Arkansas. The mortality rate from stroke in the stroke belt is about 20% greater than the rest of the nation. Through a computation by the American Heart Association (AHA), it is estimated that in 2030, the incidence of stroke will increase 21.9% in its prevalence in the U.S with an estimated 4 million more people suffering from stroke[1]. The cause of stroke is multi-factorial and there are several risk factors that are associated with stroke.

3. Risk Factors

The risk factors for that are most commonly associated with ischemic stroke are hypertension, diabetes mellitus, cigarette smoking, heart rhythm disorder, high blood cholesterol and lipids, lack of physical activity and family history and genetics.

High blood pressure is a strong determinant of risk for both ischemic and hemorrhagic stroke. High blood pressure is defined by a systolic blood pressure greater than 140 mmHg and diastolic blood pressure greater than 90 mmHg. This can lead to a blockage of the vessel (usually the middle cerebral artery) resulting in ischemia. 77% of those who have their first stroke have blood pressure greater than 140/90 mmHg[1]. The increased blood flow from high blood pressure can cause damage to artery walls and also cause atherosclerosis, or the thickening of the arterial walls making them weak and

inflexible. Diabetics with a blood pressure of less than 120/00 mm Hg have approximately half the risk for stroke compared to those with high blood pressure. Lowering the blood pressure in hypertensive diabetics through treatment significantly reduces their risk of stroke[3]. An AHA calculation estimates that high blood pressure will be up 7.2% in 2030 from 2013 estimates[4]. In 2009, the direct and indirect costs of high blood pressure were $51 billion and estimated to be $343 billion in 2030. One in three U.S. adults have high blood pressure and about 6% of people are estimated to have undiagnosed high blood pressure.

Similarly, high blood cholesterol and lipids are another risk factor for stroke. High cholesterol can increase plaque build-up in the arteries and lead to atherosclerosis. In the U.S. in 2010, 31.9 million adults that were 20 years old or older had serum cholesterol levels of ≥240 mg/dL reflecting 13.8% of adults in the U.S[1]. Diabetes mellitus is another risk factor for stroke. 19.7 million people age 20 and over are diagnosed with diabetes. Some of the long-term diabetes complications involve vascular damage thus increasing the risk of cardiovascular disease such as stroke. The strong prevalence of ischemic stroke the U.S. is due to the several contributing factors prevalent in the American population.

4. Treatment

The treatments available for ischemic stroke are very limited. The only FDA-approved drug treatment is tissue plasminogen activator (tPA), a thrombolytic agent that dissolves the arterial clot allowing the return of blood flow. However, the use of tPA is limited because it is quite time-sensitive and must be administered soon after a stroke

occurs. A newly adopted time window expansion from American Heart Association (AHA)/American Stroke Association (ASA) recently expanded the treatment window slightly. The AHA/ASA recommends that tPA can be administered within 3-4.5 hours when previously it had been strictly before 3 hours[5]. The Safe Implementation of Thrombolysis study which compares cohorts of patients that received tPA less than 3 hours after stroke and those that received it within 3-4.5 hours after stroke and found that there was no difference in outcome[6]. However, administering the drug after the recommended window increases the risk of hemorrhagic conversion, or the conversion of ischemic stroke into hemorrhagic stroke exacerbating the injury. Another tPA safety study indicates that even with appropriate administration within 3 hours, there was still a hemorrhagic risk where the symptomatic intracerebral hemorrhage rate was 5.2%[7]. tPA is efficacious when administered in the appropriate timeframe, however only a very small percentage of stroke sufferers overall actually receive the drug. In 2004, about 1.8% to 2.1% of acute ischemic stroke patients received tPA, and this rate had doubled between 2005 and 2009[8]. Overall, an estimation of only 3.4% to 5.2% ischemic stroke patients received thrombolytic treatment in 2009[8]. The reasons for the low administration of tPA remain unclear. Evidence shows that only 22% of ischemic stroke patients that showed up in the emergency department arrived within 3 hours[9]. At the time of this study between July 1993 and June 1994, 3 hours was still the upper limit for tPA administration. Some reasons for delayed arrival to the emergency department include the lack of knowledge about the warning signs of stroke and stroke treatments[10]. Also, delays are due to transportation delays to hospital emergency medical services[11]. These limitations combined preclude a vast majority of stroke patients from actually receiving

tPA[12]. Therefore, a new stroke treatment that can be administered at a delayed or extended time point could provide treatment to more stroke sufferers.

Hundreds of drugs had shown promise in preclinical animal stroke models, but had failed clinical trials as effective stroke treatments[13,14]. One such neuroprotective drug that was undergoing clinical trials was erythropoietin (EPO), a hematopoietic hormone first used as a therapy to stimulate red blood cell generation as a treatment for anemic patients. It has been used safely for this purpose for two decades and is well tolerated and commonly used. Numerous laboratory studies have championed the neuroprotective effects of EPO in stroke models[15], however its use in clinical stroke studies have engendered thromboembolic complications[16] and also made researchers question whether or not EPO has any overall remarkable effect on stroke. Thus, although preclinical studies may prove to be successful, not all studies succeed in clinical trials demonstrating that any new treatment must satisfy many criteria before actual use in the clinic for treating stroke.

The field of stroke therapy research spans a broad range of approaches including the investigation of pharmacological agents for neuroprotection and thrombolysis, adaptive physical rehabilitation, stem cell transplantation, transcranial magnetic stimulation, and trophic factor treatments. The search is underway for new candidates for treating stroke including strategies to maximize endogenous repair with trophic factors and stem cell transplantation. New therapeutic approaches are being developed to enhance tissue regeneration and reduce cell death in the ischemic brain area, thereby preserving neuronal function and decreasing the disability of stroke sufferers.

B. Ischemic Injury Cell Death Mechanisms

1. Heterogeneous Cell Death

In order to elucidate new targets for therapy, it is necessary to understand the mechanisms of cell death from ischemic injury. First we must understand that neuronal tissues are physiologically more susceptible to injury from ischemia compared to other tissues in the body due to their high metabolic demands and relatively low storage supply of glucose or glycogen metabolic substrates[17]. Next to the heart and the kidney cortex, the brain has one of the greatest energy demands resulting in a high resting metabolic rate[18]. Although the rate of cell death varies among cell types and regional populations of neurons, these cells typically undergo rapid cell death within minutes of ischemia and delayed cell death hours to days later. The disability and mortality rendered by stroke is associated with neuronal death and loss or compromise of a regional brain function.

Ischemic injury inflicts multiple stresses to the brain parenchyma. The vessel can no longer provide oxygen, glucose or other metabolic substrates to the brain tissue. A cascade of molecular, cellular and systemic events is initiated from the onset of the ischemic insult. Ischemic injury results from heterogeneous mechanisms because it can be characterized by different modes of cell death. Each mode is broadly categorized into two different time-dependent phases of injury: the initial acute phase and the delayed, or chronic phase of stroke. In the acute phase, cell death occurs within minutes of ischemia and extends to hours beyond the initial event. The acute phase is characterized generally by necrotic cell death by cellular swelling and bursting of the plasma membrane due to the sudden inability to maintain ionic homeostasis during ischemia. This type of injury

creates the core of the damage, an area where the reduction of blood flow and cell death is most severe.

The later delayed phase of cell death occurs from hours to days to weeks after a stroke event spreading the injury to areas further from the initial damage. The delayed phase occurs as a consequence of the release of injurious contents of damaged cells from the initial injury, including excitatory amino acids and proteases into infarct and peri-infarct areas. The progression of inflammation and the weakening of the blood brain barrier potentiate the damage. This peri-infarct region is defined as the ischemic penumbra. The reduction in blood flow in this area is not as severe compared to tissue of the infarct core due to the supply from residual or collateral blood flow. Even though the neurons in the penumbra undergo disregulation, they may not undergo immediate cell death[19]. The secondary phase of cell death comprises mainly of a programmed cell death, apoptosis. The aim for this section of the background is to illustrate the heterogenous nature of cell death after ischemia and the wide spectrum of mechanisms. Understanding ischemia as a heterogenous injury allows researchers to target such pathways for treatments.

2. Necrosis

Necrosis a mode of cell death and is a rapid pathological response to severe cellular insults like hypoxia or ischemia. There is an overlap and transition from the acute to delayed events after injury. Generally, necrosis is associated with the acute phase of ischemia and peaks around 8 hours[20]. Some consider necrosis to be an explosion of the cell and its contents and apoptosis, an implosion of the cell. Necrosis is a mode of cell

death that spills cellular contents into the extracellular space causing inflammation in the surrounding tissue by attracting immune cells such as microglia, neutrophils and monocytes perpetuating the injury. In contrast, apoptosis is classically considered to be a programmed implosion of the cell. The cell shrinks upon itself resulting in minimal inflammation and effects on the surrounding tissue. Apoptosis can be considered to be cellular suicide whereas necrosis is cellular homicide. Apoptosis is a passive cell death caused by an injury. Even though we have thought of necrosis as the alternative to apoptosis, recently scientists are now investigating necrosis as a regulated cell response and a possible programmed death with a regulated role in physiology[21]. The differences between apoptosis and necrosis are highlighted in the table here.

Necrosis	Apoptosis
Cell "explosion"	Cell "implosion"
Increases inflammation in surrounding tissue	No inflammation to surrounding tissue
Cell swelling, loss of membrane integrity, lysis	Cell shrinkage, intact membrane, cell fragmented into smaller bodies
No phosphatidylserine translocation	Phosphatidylserine translocation for signaling phagocytosis
Random DNA fragmenting- The random fragmenting results in a DNA smear pattern on electrophoresis gel	Predictable DNA fragmenting- Fragmentation is characteristically found at internucleosomal sites where endonucleases cleave 180-200 bp pieces producing a ladder pattern visualized with electrophoresis[44,45]
No requirement of energy	Requires energy, ATP-dependent process

Table 1.1. Differences between necrosis and apoptosis. Necrosis and apoptosis are two different modes of cell death found heterogeneously in the ischemic injury. Upon the initiation of the ischemic event, cells in the area are immediately are starved for oxygen, glucose, and other metabolic substrates causing cell death soon after resulting in heterogeneous cell death.

Necrosis occurs when the ionic homeostasis has been disrupted and cell swelling occurs. Necrosis occurs after oncosis, which is derived from ónkos meaning “to swell”. Excitotoxicity and overstimulation of the glutamate receptors plays a big role in necrosis by disrupting the ionic homeostasis of Ca^{2+} and Na^{+} which is associated with Cl^{-} influx leading water into the cell and causing swelling and bursting[22,23]. Morphological indicators of necrosis include cell swelling, late DNA fragmentation, dilation of the mitochondria, and rupturing of organelle membranes, and the nuclear and plasma membranes[23].

When the ischemic insult is not severe enough to elicit necrosis, apoptosis may occur. Necrosis is an immediate response to an extreme insult, whereas apoptosis is a delayed response genetically programmed and spares neighboring cells from subsequent inflammation. An insult may not be great enough to cause necrosis, or the tissue may be affected in a delayed fashion allowing a cell enough time to trigger a genetically-programmed death[23]. Classically, necrosis had been considered to be independent of protein synthesis and caspase mechanisms. Necrosis is different than apoptosis, which has been considered to be caspase-dependent[24]. Necrosis and apoptosis are distinguishable cell death events each with different morphologies; however, recently these events have been considered to be on a continuum of cell death with overlapping triggers and mechanisms rather than diametrically opposed modes of death[25]. It is certainly possible for the two cell death mechanisms to be set off by the same triggers, but cells that undergo apoptosis most likely faced a milder insult rather than cells that undergo necrosis that faced a harsher insult. Both modes of cell death can be activated by the same triggers like cytokines, pathogens, heat and ischemia[21]. More recent data has

demonstrated that they can both converge in final cell death pathways releasing cytochrome c and activating caspase-3[24]. The mechanisms to prevent apoptosis such as Bcl-2/Bcl-xL and heat shock proteins have been able to prevent necrosis as well[21]. This provides evidence that necrosis can also be a programmed cell death. It has long been thought that necrosis was not genetically regulated, in which a swelling of the cell cytoplasm and mitochondria was the only cause death. However some groups elucidated that a quick but organized caspase-9-dependent caspase-3 execution occurs also during necrosis[24]. Inhibitors of caspase-9 and -3 resulted in a reduction of necrotic neurons[24]. In ischemic injury, necrosis and apoptosis can share a final common pathway of caspase-3 due to hypoxia affecting the mitochondria releasing cytochrome c to initiate caspase activity[24]. Our paradigms regarding necrotic and apoptotic mechanisms have shifted significantly into overlapping modes of cell death as more cellular and molecular evidence has come to light.

3. Apoptosis

The term "apoptosis" was introduced by Kerr et al. in 1972 as a "controlled cell deletion" that is part of both pathological and normal physiological processes[26]. Developmentally, apoptosis occurs to prune new neurons in a young brain and to eliminate the cells of our embryonic webbed hands[26]. Apoptosis is an execution of genetic programs. It is a mode for the cell to eliminate itself without imposing harm to the surrounding tissue. There are several characteristics of apoptosis including chromatin aggregation, DNA fragmentation, cell membrane blebbing, the creation of apoptotic bodies, shrinking cell volume, phosphatidylserine translocation, and mitochondrial

depolarization. Apoptotic cells morphologically display condensation/shrinkage of the nucleus and cytoplasm to create "apoptotic bodies". These bodies isolate fragments of the cell into membrane-bound vesicles, which are extruded to be phagocytosed by the surrounding cells and degraded by lysosomes[26].

Phosphatidylserine translocation is one of the downstream characteristics of apoptosis. The plasma membrane of apoptotic cells signals to phagocytes that they are apoptotic through the translocation of phosholipid phosphatidylserine (PS) to the outer leaflet of the plasma. In healthy cells, PS is sequestered in the inner leaflet of the plasma membrane. Reports have demonstrated that phagocytosis cannot be initiated by L-isoforms of molecules similar to PS such as glycerophosphorylserine and phosphoserine, but that PS in the outer plasma membrane leaflet is specifically necessary for the recognition of apoptotic cells by phagocytes[27]. PS membrane externalization was inhibited by the overexpression of Bcl-2 or Abl[28]. PS translocation inhibition prevents phagocytosis of the apoptotic cells. Although the PS translocation mechanism is accepted as a classic signal of apoptotic phagocytosis, there are also other surface markers that indicate an apoptotic cell. For example, certain populations of macrophages use the vitronectin receptor to recognize apoptotic elements for phagocytosis[29].

DNA fragmentation is another characterization of apoptosis. This fragmentation is characteristically found at internucleosomal sites where endonucleases cleave 180-200 bp pieces producing a ladder pattern visualized with electrophoresis[30,31]. Although DNA is fragmented in necrotic cells as well, it is a random breakage of the DNA. This appears as a vertical band in a smear pattern with electrophoresis. It's debatable as to whether or not DNA fragmentation alone suffices as the only criterion to identify apoptosis[32,33]. Defining

apoptotic cells based on DNA cleavage remains controversial. Necrotic cells too have been demonstrated to produce an apoptosis-like DNA signature[32]. Others have observed apoptotic-like DNA signatures in non-dying cells and concluded that they are artifactual[33]. Single-stranded nicks of DNA has also been observed with apoptotic cells[34]. These morphological features of apoptosis should be taken into consideration when evaluating dying cells. These hallmark necrosis and apoptosis events are necessary to understand as markers to qualify and quantify cell death. Apoptosis may be induced through the extrinsic or intrinsic pathways. Both pathways converge at the end stage of caspase activation. These pathways share common downstream effectors of apoptosis, however the way in which the apoptotic pathway is activated differs through intrinsic or extrinsic activation.

a. Intrinsic Pathway

Intrinsic apoptotic activation arises from endogenous cellular stimuli such as insults to the mitochondria due to DNA damage or oxidative stress. The intrinsic apoptotic pathway is also stimulated by insults from ischemia such as ionic disregulation including an influx of Ca^{2+} into the cytosol of the cell. Disregulation occurs when glutamate binds to AMPA or NMDA receptor increasing Ca^{2+} entry into the cell. The increased Ca^{2+} stimulates calpains to mediate the truncation of Bid to the truncated form, tBid. tBid interacts with proapoptotic proteins, Bad and Bax which are normally neutralized by Bcl-2 and Bcl-xL under non-apoptotic conditions[35]. The heterodimerization of the proapoptotic proteins with tBid stimulates the mitochondria to release cytochrome c or apoptosis inducing factor (AIF) to enact the caspase pathways.

Cytochrome c contributes to the activation of the Apoptotic Protease Activating Factor 1 (APAF-1). Apaf-1, cytochrome c, and pro-caspase 9 form a complex known as the apoptosome, which leads to the activation of caspase-9. Caspase-9 activates caspase-3, a death-executing caspase. Inhibition of the APAF-1 pathway during hypoxia-ischemia reduces caspase-9 and caspase-3 activation and thus attenuates brain injury[36]. Other than cytochrome c, the mitochondria can also release AIF to translocate directly to the nucleus to activate large-scale DNA fragmentation and inducing apoptosis in a caspase-independent manner. During apoptosis, the cell requires energy for tasks like the chromatin and cytoplasmic changes that occur during apoptosis. Not all these tasks require energy, but if no energy is available, cell swelling and necrosis occur[37].

b. Extrinsic Pathway

The extrinsic apoptotic pathway, also known as the death receptor pathway is activated by signals from the extracellular space binding to the membrane death receptors of the cell. Such signals include inflammatory factors like cytokines which are small proteins that mediate inflammation during trauma, infections and ischemic injury. Cytokines such as Fas ligand, tumor necrosis factor (TNF) and lymphotoxin alpha, tumor necrosis factor-like weak inducer of apoptosis (TWEAK), and TRAIL are upregulated after ischemia and bind to their respective receptors: Fas (CD95/Apo-1), TNF receptor 1, TRAIL-R1, TRAIL-R2, Fn14. For example, Fas ligand binds to the Fas receptor and complex with Fas associated death domain (FADD) and pro-caspase-8 to create a death-inducing signaling complex (DISC). Activated caspase-8, an initiator caspase can

mediate truncation of Bid to tBid to activate mitochondrial pathways and or it can directly activate caspase-3, an executioner protein enabling cell death[35].

a. Commonality in Both Pathways: Caspase Activation

Intrinsic and extrinsic apoptotic pathways converge on the final step of caspase activation. Caspases are cysteine proteases necessary for propagating and executing apoptosis in ischemic injury, particularly caspase-3. It has been demonstrated that inhibiting caspase with a pan-caspase inhibitor was neuroprotective 3 hours after ischemia in a rat ischemic stroke model[38]. Caspases execute apoptosis by activating other caspases, dissembling the cytoskeleton, inactivating anti-apoptotic factors, and caspase activating DNAase (CAD) for the fragmentation of DNA. Caspase activation also leads to cleavage of nuclear laminins, actin and other cytoskeletal proteins. There are several factors that contribute to cell death after ischemia including the disregulation of ionic homeostasis and excitotoxicity. Both ionic disregulation and excitoxicity contribute to necrosis and apoptosis are highlighted here.

4. Ionic Homeostasis

The loss of ionic homeostasis in neurons is a major consequence of ischemic insult. Because of the sensitive nature of ionic homeostasis in the neuron, any disruption may cause apoptosis[39-41]. The electrochemical gradients maintained by neurons are essential to their function particularly in firing action potentials. These gradients are characterized by the unequal distribution of Na^+, K^+, and Ca^{2+} between the extracellular and intracellular spaces. These gradients are normally maintained by voltage-gated

channels, ion exchangers, ion pumps, ion transporters, and ATPases. Maintenance of these essential gradients requires 50-60% of the total ATP produced in the brain[42]. Reduction in the energy supplying processes during brain pathologies, such as ischemia, hypoxia or hypoglycemia, results in a rapid loss of ionic gradient homeostasis leading to abnormal Na^+ and Ca^{2+} influx and K^+ efflux. Neurons maintain a negative membrane potential, and the loss of this ionic distribution can result in the abnormal depolarization of the membrane. The ionic concentrations do no act independently of each other, thus when one electrochemical gradient is disrupted, the other ionic gradients are consequently affected.

Hypoxia and ischemia increase the intracellular Na^+ concentration and can lead to neuronal cell death[43,44]. This is due to the activation of voltage-sensitive sodium channels during hypoxia or ischemia[45]. With the extracellular removal of Na^+ or blocking of Na^+ channels with tetrodotoxin (TTX), cellular injury is reduced[46-48]. Since ion gradients are not independent of each other, the experimental in vitro addition of Na^+ has consequences for the Ca^{2+} gradient which can be fatal to the cell[49]. Addition of Na^+ caused intracellular Ca^{2+} to rise and the cells swelled and died[46].

Ischemia affects K^+ ion homeostasis as well. Ischemia initiates depolarization which results in a loss of intracellular K^+ concentration. The reduction of intracellular K^+ induces apoptosis[41]. Efflux of K^+ from the delayed rectifying current, I_K revealed to be apoptotic even when raises in intracellular Ca^{2+} were prevented. Applying the K^+ blocker, tetraethylammonium (TEA) attenuated K+ efflux reducing further cell death and blocking other K^+ channels, Ca^{2+} and Cl^- channels could not reduce apoptosis[41]. Cellular K^+ efflux leads to cellular shrinkage. Antagonizing the NMDA receptor or raising extracellular K^+

reduces cell body shrinkage and cell death[41]. Because excessive K^+ efflux is thought to contribute to ischemia-induced cell death, Wei and colleagues were able to attenuate cell death by administering the K^+ channel blockers, TEA and clofilium in vivo before stroke[50]. Ionic disregulation can also lead to cell death via glutamate excitotoxicity.

3. Excitotoxicity

Excitotoxicity is also another major cause of cell death after ischemia. Glutamate is the major excitatory neurotransmitter in the central nervous system and is released from neurons during ischemia. Glutamate can bind to three types of receptors: N-methyl-D-aspartate (NMDA), DL--amino-3-hydroxy-5-methylisoxazole-propionic acid (AMPA), and kainate receptors[51]. The NMDA receptor is associated with a membrane channel that is selectively permeable to Na^+ and Ca^{2+}. Under ischemic conditions, there is a great increase in extracellular glutamate representing the initial synaptic release of glutamate[52,53]. Disregulation occurs when glutamate binds to the NMDA receptor which leads to an influx of Ca^{2+} and Na^+ through associated channels potentiating excitotoxicity[22,51]. Increased intracellular Ca^{2+} is a major type of ionic disregulation that causes cell damage or death. Studies have demonstrated that neuronal cell death is attenuated by blockading NMDA receptors in vitro with the post-synaptic excitatory amino acid blocker gamma-D-glutamylglycine (DGG)[54,55]. Some neuronal populations are more vulnerable to excitotoxicity than others, such as the neurons of the CA1 region compared to the CA3 region of the hippocampus. The CA1 neurons exhibit much greater NMDA receptor-dependent Ca^{2+} influx, reactive oxygen species, and mitochondrial dysfunction than CA3 neurons under in vitro ischemia[56].

This illustrates the role of the NMDA receptor and excitatory amino acids in propagating neuronal injury. Consequentially the passive entry of water and Cl^- ions into the cell ensues to cause swelling within the first few minutes of the glutamate insult[57]. Since ischemia impairs the ability of cells to generate sufficient energy to maintain ion gradients, the internal concentration of Ca^{2+} increases. Further exacerbating the problem, elevated intracellular calcium stimulates the release of internal stores of calcium from the endoplasmic reticulum[58]. Many cellular enzymes that are normally regulated by cytosolic Ca^{2+}, including the calcium-activated proteases calpains, lipases, and protein kinase C are disregulated when when Ca^{2+} is disregulated. Excess Ca^{2+} can lead to catabolic processes, the production of reactive oxygen and nitrogen species, and energy depletion through activation of Ca^{2+} ATPases in the cell[59].

C. Experimental Models of Stroke

1. In Vitro Models

There are several ways to model the modes of cell death and ischemic injury in vitro and in vivo to elucidate the pathophysiology of stroke. In vitro models allow for a molecular and cellular scope of experimentation and study. Oxygen Glucose Deprivation (OGD) is a common method used to mimic ischemic conditions in vitro by depriving cell cultures. Primary neurons or mixed cultures of neurons and glia are deprived of the metabolites, glucose and oxygen in this model. In brief, cell culture growth media is replaced with a buffered physiological solution such as Ringer's solution and placed in an airtight hypoxia chamber (0.1-0.3% oxygen) for a designated amount of time. The amount of time for the hypoxia treatment will depend on the cell type and the

degree of insult required. After hypoxic treatment, the cultures are returned to normoxic conditions and cell death or survival is assessed during this reoxygenation period. The reoxygenation period mimics the reperfusion phase of stroke.

2. In Vivo Stroke Models

a. Focal Ischemia Stroke Models

The middle cerebral artery occlusion (MCAO) model in animals is commonly used to model focal ischemic stroke. It simulates human stroke conditions since most ischemic strokes in humans are due to a MCAO. One of the earlier methods for inducing stroke is occluding the proximal MCA in the rat which causes ischemic damage to the basal ganglia and cortex[60,61]. This procedure requires a subtemporal craniotomy. To contrast, another method is the intraluminal method which is less invasive and requires no craniotomy[62]. An incision is made to the neck and a nylon intraluminal suture is passed through the external carotid artery occluding the MCA. This procedure can be varied with a thicker (2-0) or thinner (4-0) suture thread[62,63].

The model of stroke used in this dissertation is the whisker barrel cortex ministroke, which is a variation of the MCAO model that induces stroke in the whisker barrel cortex. Three to six branches of the MCA around the barrel cortex were identified and then occluded with sutures[64]. Wei et al. described using this method in the rat[64]. Here, the vasculature of the barrel cortex was visualized using intrinsic optical signal imaging. Blood flow in the barrel cortex can be evoked by stimulating the contralateral whiskers. Targeting a distinct somatosensory area with a defined function lends itself for studying

the stroke pathology in this area. Further, the animal's blood flow, functional recovery, and rehabilitation before and after stroke can be tested.

The functionality of the whiskers can be tested by observing the behavior associated with the sensitivity of the whiskers after stroke. For example, the corner test can be used to test whisker function[65]. The mouse enters a 30° angle corner so that both sides of whiskers are stimulated deep in the corner[65]. Stereotypically, unaffected animals turn around both left and right ways equally. If stroke was induced on the right side of the brain, the animal will turn stereotypically towards the ipsilateral side more than the deficient contralateral side. The whisker barrel ministroke model is also useful for testing rehabilitation therapy models using whisker stimulation to provide afferent thalamocortical signals to the affected barrel cortex[66]. Studies show that providing these stimulating signals to the stroke area can improve function[66,67].

Focal ischemia can be induced in other ways such as the photothrombotic, autologous clot, and endothelin-1 methods. The photothrombotic method is a non-invasive method in which a photosensitive dye is intravenously injected into the animal[68-70]. To induce thrombotic occlusion, the distal MCA is irradiated by a laser to activate microthromboses. This model is not very invasive and it allows for MCAO in awake animals. The autologous clot model also simulates thromoboembolic stroke. In this technique, the embolus is created by removing the arterial blood from a donor into tubing of predetermined size. The clots are formed by alternating the blood through different syringes with saline. The clot can be introduced into the external carotid artery (ECA) to be lodged into the origin of the MCA[71]. However, thromboembolic models do not produce consistent infarcts or allow for precise control of reperfusion[71,72].

In the Endothelin-1 model, the vasoconstrictor peptide Endothelin-1 (ET-1) is injected into the brain. This peptide reduces blood flow and induces ischemic injury[73]. This model can induce transient to semi-permanent focal ischemia[74]. ET-1 can be injected intracerebrally or topically applied onto the brain into an area of interest[73]. It can also be injected next to the MCA to create a larger ischemic injury[74].

b. Global Ischemia Stroke Models

Global ischemia differs from focal ischemia in that the whole brain experiences ischemia due to a halt or dramatic reduction in blood flow such as from cardiac arrest or suffocation. In the two-vessel occlusion model, the bilateral occlusion of the CCA paired with systemic hypotension (down to 50 mm Hg) attenuates blood flow to the forebrain and induces ischemic damage to the forebrain[75]. Systemic hypotension is created by arterial exsanguination and clamping of the CCA. The occluding clamps are then removed and the drained blood is used for brain reperfusion[76]. Varying the duration of ischemic conditions will cause damage to different brain areas. This occlusion technique cannot be used in awake animals, but it produces a reversible and high-quality forebrain ischemia.

E. Summary

Stroke is a devastating disease affects people worldwide. Ischemic stroke has a complex pathophysiology initiated by several events in close succession upon vessel occlusion such as ionic disregulation and excitotoxicity resulting in a heterogenous mix of cell death. This chapter provided a background of the epidemiology and treatment of

stroke, the intracellular mechanisms of necrosis and apoptosis, and emphasized the importance of the research tools used to understand the pathophysiology of stroke such as in vitro and in vivo stroke models. A comprehensive understanding of stroke will help us uncover more regenerative therapeutics. The following chapters will focus on the regenerative aspect of the brain after stroke including endogenous migration and stem cell transplantation.

Chapter II

Endogenous Regeneration After Stroke

The adult brain has the ability for endogenous regeneration after a stroke as an attempt at self-repair[77-80]. As a physiological process, the neural progenitors from the subventricular zone (SVZ) migrate in rostral migratory stream to the olfactory bulb to differentiate into functional interneurons[81]. Upregulated neurogenesis and neuronal progenitor migration to the infarct have been repeatedly observed in the rodent and human subventricular zone (SVZ) following cerebral ischemia[66,78,82]. However, many of these neural cells do not survive upon reaching the injury site due to the cytotoxic microenvironment of the infarct[83]. On its own, endogenous neural progenitor proliferation and migration cannot sufficiently repair damaged ischemic tissue and restore function[84]. This chapter focuses on how chemoattractive factors such as chemokines play definitive roles in neural progenitor recruitment, proliferation, migration, and limited tissue replacement after stroke.

A. Rostral Migratory Stream Migration

Regenerative activities in the adult brain was first observed over 30 years ago[85]. Now it is commonly accepted that endogenous neurogenesis occurs in adult mammalian brain contradicting the old dogma about the brain's inability to regenerate[86]. Compelling evidence has demonstrated that there is a continuous production of neurons in adult non-human primates as well as in humans[87,88]. Under physiological conditions, adult mammalian brains have endogenous stem cells that migrate in a network of chains along the rostral migratory stream to the olfactory bulb where they differentiate into interneurons and are incorporated into existing neural circuitry[89,90]. The reasons for the renewal of these olfactory bulb cells are not clear. This migratory stream can be also

directed to infarcted areas and help in repairing damaged brain tissue. Specific regions of the adult brain such as the SVZ and the subgranular zone (SGZ) are capable of responding to injurious signals from other areas of the brain by increasing the proliferation of neuroblasts[78,91]. This proliferative response also occurs in response to non-injurious stimuli such as in the SGZ after exercise[92,93]. This response and the migration of newly formed neural progenitor cells towards the injured tissue are regarded as a part of repair process in the adult brain. So far, numerous factors have been identified of being capable of enhancing neural progenitor proliferation and neuroblast migration after a cerebral ischemic insult[94].

B. Inflammation and Regeneration

Inflammatory factors are important in the repair of stroke tissue. Even though inflammation is a significant cause of cell death in stroke, it can paradoxically play a role in neurogenesis, migration and regeneration. Pro-inflammatory factors such as TNF-α can increase the proliferation and recruitment of endogenous neural progenitors to the ischemic infarct[95]. These factors can also enhance progenitor survival, and induce the migration of neuroblasts from the SVZ towards the site of ischemic injury[78,82,95].

Interestingly, numerous chemoattractive agents upregulated by ischemia and inflammation can be classified as both pathologic and therapeutic depending on the time course and the extent of their expression[96]. Matrix metalloproteases (MMPs) upregulation, for example, is highly detrimental in the acute phase of stroke, however, MMPs are also upregulated by cells migrating from the SVZ to the ischemic penumbra[97]. MMPs in the chronic phase of stroke are involved in regeneration and tissue repair of the

damage[97]. Blocking MMPs in the weeks following injury significantly decreases neural progenitor migration and is detrimental to functional recovery[98,99]. Additionally, factors such as monocyte chemotactic protein-1 (MCP-1) secreted by astrocytes and microglia not only serve as chemoattractants for lymphocytes, basophiles and macrophages, but also neural progenitors[100]. Knocking out MCP-1 expression significantly decreases neuronal progenitor migration in vivo[101]. These inflammatory factors have dynamic reparative roles in stroke and migration.

New studies have revealed that the role of inflammation on neurogenesis is much more complex than previously thought. Some aspects of inflammation can support neurogenesis, differentiation, proliferation, migration, and survival[102]. Inflammatory reactions especially from microglia can be beneficial after injury. Recent studies have shown that neuroinflammation and microglia can have beneficial effects on neurogenesis[102,103]. In a co-culture system of neural stem cells and microglia or growing stem cells in microglia-conditioned media, a protective effect on stem cells was observed; neural stem cells in conditioned media survived longer than those of control cultures[104].

Inflammation also provides guidance cues. Inflammatory cues direct the migration of repair progenitors. Both transplanted and endogenous neural progenitors have been shown to migrate to the site of neuroinflammation and chemokines. For example, microglia found at the injury are a source of cytokines like IL-6, TNFα, and IL-10 and also a source of neurotrophins like brain-derived neurotrophic factor (BDNF), and other chemokines[105,106]. In a study where conditioned media from microglia cultures were applied to precursor cells and assayed for its effects on migration and differentiation, an increase in migration with microglia-conditioned media was seen in the Transwell

migration assay[104]. Similarly, when an inflammatory stimulus was injected into a hippocampal slice culture, green fluorescent protein (GFP)-labeled neural progenitors transplanted in the slice migrated towards the site of the inflammatory stimulus 7 days after injection[95]. The injection consisted of TNF-α and interferon-γ, the bacterial toxin, LPS, the human immunodeficiency virus-1 coat protein glycoprotein 120, or a β-amyloid-expressing adenovirus. This suggests that cytokines from sites of neuroinflammation can attract neural progenitors towards the inflammatory gradient[95]. The ischemic lesion is a reactive area with inflammation, cell death, and also several chemoattractants. Even though inflammatory cells have a neurotoxic role, work also supports that inflammatory cells can also play a role in neuroprotection and reparative progenitor migration.

C. SDF-1α Plays a Chemoattractive Role in Stroke

1. SDF-1α is a Chemokine

Chemokines are chemoattractive cytokines that are expressed both in the peripheral nervous system and the CNS. Chemokines have been mainly associated with inflammation. For example, they are greatly involved in neutrophil chemotaxis during the inflammatory process. However, they also recruit embryonic, adult progenitor, and cancer cells. It is clear now that directed cell movement in developmental and pathological processes rely heavily on chemokines.

Stromal cell-derived factor-1α (SDF-1α) is constitutively expressed chemokine acting mainly through G-coupled protein transmembrane receptors such as CXC chemokine receptor-4 (CXCR4)[107,108]. In the peripheral system, SDF-1α is highly

chemoattractive for lymphocytes and it directs the migration of hematopoietic cells from the fetal liver to the developing bone marrow[109,110]. SDF-1α is a leukocyte localizing cue for leukocyte entry into the CNS parenchyma[111]. The CXCR4 receptor has other functions besides inflammatory cell chemotaxis[112]. For example in development, SDF-1α knockout mice have impaired cerebellar neuronal migration where the external granule cell layer develops irregularly[110]. Similar abnormalities such as impaired myelopoiesis, cardiogenesis, and β-lymphopoiesis arise in both SDF-1α and CXCR4-deficient mice illustrating that SDF-1α and its receptor are involved in other functions aside from the immune system[110,113].

Like many chemokines, SDF-1α plays a role in cell trafficking and is a potent chemoattractant to many cell types. CXCR4 is expressed in many progenitor cell types including hematopoietic stem cells[114], skeletal muscle satellite progenitor cells[115], neural progenitors[116], hepatic oval cells[117], as well as retinal pigment epithelium progenitors[118]. CXCR4 is also expressed in neurons (Figure 2.1). The SDF-1α/CXCR4 axis plays a role in trafficking organ progenitor cells during development for embryogenesis, organogenesis, tissue and organ regeneration in adulthood, and in cancer metastasis. SDF-1α and CXCR4 expression can be found in the stem cells derived from the inner cell mass of the blastocyst and SDF-1α increases the survival and migration of these cells[119]. In adults, to further illustrate the chemoattractive

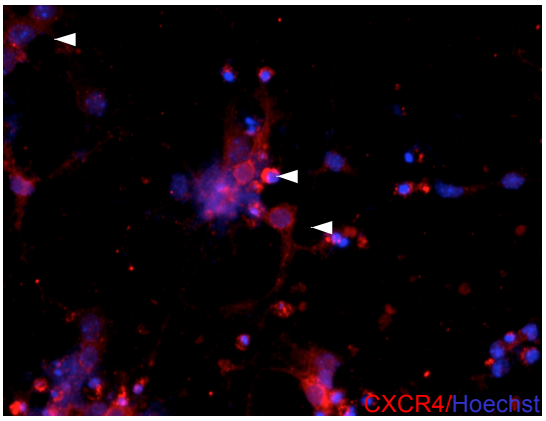


Figure 2.1. CXCR4 receptors are expressed on cortical neurons in vitro. These are one of the many cell types that express CXCR4. White arrows indicate positive staining of cytoplasmic CXCR4.

abilities of SDF-1α, meninges from SDF-1α-deficient mice were transplanted onto host mice and found to exhibit no attraction of neural progenitors to the transplant whereas wild-type animal meningial transplants exhibited progenitor migration in the host[120]. When the subventricular neural stem cells were infected with a CXCR4 siRNA retroviral vector, migration of these progenitors was attenuated compared to the empty control vector[121]. These examples illustrate that SDF-1α/CXCR4 signaling plays migratory roles in both embryonic and adult systems.

2. SDF-1α and Ischemia

Chemoattractive factors such as SDF-1α are highly expressed in the infarct after ischemic stroke and divert neural progenitors from the rostral migratory stream to the infarct[66,122,123]. Neuronal progenitor migration begins within hours after injury, peaks at approximately 3 weeks following the focal ischemic event[124-126]. Cerebral ischemia greatly increases SVZ neural progenitor cell proliferation, and progenitor migration to the infarct and can elicit a 31-fold increase in proliferation of progenitors that differentiated into neurons at the infarct[78,125,127-130].

We and other groups have shown that SDF-1α is upregulated in the infarct and peri-infarct area in a gradient-fashion with SDF-1α strongest in the core[131-133]. Its expression peaks at 7 to 14 days after the insult[134-136]. Robin and colleagues demonstrated that neural progenitor cells migrated 67.2 μm after ischemic stroke compared to the control in which progenitors migrated 25.2 μm[132]. When neutralizing SDF-1α with an antibody, migration was attenuated[132]. SDF-1α increases cell survival through increasing the expression of anti-apoptotic proteins. Its neuroprotective protective effect has been

demonstrated in vitro and in vivo. Cells exposed to hydrogen peroxide (H_2O_2) exhibited fewer caspase-3-positive cells and more Bcl-2 expression when SDF-1α was added[137]. Similarly, when SDF-1α protein was injected into the ischemic brain, infarct size decreased and motor function increased demonstrating that SDF-1α contributes to neuroprotection and repair in the brain[137].

SDF-1α is produced and released by astrocytes and endothelial cells from the tissues surrounding the ischemic injury[134]. SDF-1α is upregulated by activated astrocytes and vascular endothelial cells during ischemic injury[82,132,138,139]. SDF-1α works through binding its membrane-spanning G-protein coupled receptor to initiate divergent cascades leading to chemoattraction, survival, cell proliferation, and gene transcription[140]. Upon ligand-receptor binding, the Gα, Gβ and Gγ subunits that are normally associated under basal conditions dissociate. Downstream phosphoinositide 3-kinase (PI3K) activates protein kinase B (AKT) which can lead to cell survival. Cell survival may also be promoted through p38 and extracellular signal-regulated kinases (ERK) as well[141]. This pathway, however, could lead to tumor cell survival as well.

Under hypoxic conditions, the transcription factor, hypoxia-inducible factor-1α (HIF-1α) is a key player in the induction of SDF-1α expression. Many adaptations occur at the cellular level in response to hypoxia including the upregulation of HIF-1α, the master oxygen sensor in the cell. HIF-1α regulates the SDF-1α gene transcription in cells, demonstrating that SDF-1α is selectively upregulated in areas of decreased oxygen tension[142]. Consequently, HIF-1α expression increased SDF-1α-induced migration of CXCR4-positive progenitors to the ischemic injury. SDF-1α was highly expressed in the ischemic tissue especially in the vasculature including the endothelial cells and the

vascular lumen[142]. In human endothelial cells in vitro SDF-1α secretion after ischemic conditions was examined. In exposing to 1% oxygen, there was a nine-fold increase in SDF-1α secretion at 12 hours[142]. Importantly, using cells transfected with a mutated HIF-1α binding site, an attenuation of hypoxia-related gene expression including SDF-1α was observed. These experiments demonstrate the close interplay of HIF-1α and SDF-1α under ischemic conditions.

3. SDF-1α as a Therapeutic

Researchers may utilize the chemoattractive properties of SDF-1α for exogenous transplanted cell types as well[143]. SDF-1α not only plays a role in the homing of endogenous progenitors from the SVZ, but can also home bone marrow stem cells and any transplanted cells expressing CXCR4 to the ischemic infarct. Endogenously, SDF-1α mobilizes hematopoietic bone marrow stem cells that express CXCR4 to the peripheral blood and then to the stroke infarct[110,137]. CXCR4 and SDF-1α draw hematopoietic stem cells from the bone marrow to the peripheral blood[144]. Rats injected with SDF-1α 30 minutes after stroke revealed more 5-bromodeoxyuridine (BrdU)-labeled engrafted cells in the ipsilateral cortex 3 days after stroke compared to the controls[137]. Similarly, GFP-positive bone marrow cells from a GFP transgenic rat injected into a donor had greater GFP cells in the penumbra after SDF-1α local injection compared to the control[137]. With SDF-1 upregulation in the penumbra, neural stem cells transplanted into the contralateral hemisphere also migrate towards the site of ischemia[82]. These results suggest that SDF-1α and its chemoattractive abilities for endogenous and exogenous stem cells provide a molecular target for a potential therapy for ischemic stroke.

D. Summary

Contrary to old dogma, the adult brain has the ability to regenerate neurons in physiological and pathological conditions. Some inflammatory factors play paradoxical roles in tissue breakdown and tissue regeneration. Such factors include TNFα, MMPs, and MCP-1 that contribute to the injury in the acute phase of stroke, but also increase the migration of neural progenitors in the delayed phase of stroke. SDF-1α is also another chemoattractive factor that is upregulated in the vessels and astrocytes of the infarct and peri-infarct after stroke via the HIF-1α oxygen sensing mechanism. SDF-1α is a potent chemokine that has the ability to attract endogenous neural progenitors from the SVZ to the stroke injury areas for tissue regeneration. SDF-1α and its receptor, CXCR4 work in conjunction to attract inflammatory and reparative cells to the injury area. The modulation or addition of SDF-1α in the stroke tissue is a promising molecular target for tissue regeneration.

Chapter 2 of this dissertation contains excerpts reprinted from a book chapter[144] with permission from Nova Science Publishers, Inc.
Neuronal Migration: Disorders, Genetic Factors, and Treatment Options, Ch. 5 Neurogenesis and Neural Progenitor Migration after Stroke, pages 115-142. Nova Science Publishers, Inc. 2012 by Chau, M, McCullough, I, Yu, SP, Wei, L.

Chapter III
Stem Cell Transplantation As a Stroke Therapy

Stem cell transplantation has been a promising avenue for the treatment of stroke based on their pleiotropic characteristics. Pluripotent stem cells especially have the ability to differentiate and replace the cells lost from stroke and are also vehicles of trophic factor delivery, thus they can enhance endogenous regeneration and provide neurotrophic factors after stroke. These therapeutic approaches may preserve neuronal function and reduce disability in stroke sufferers. The aim of this chapter is to highlight the benefits of stem cell transplantation especially induced pluripotent stem (iPS) cells, the effects that their trophic factors can have on stroke tissue and brain function, and strategies used to upregulate these factors before transplantation. This field of transplantation encompasses a wide range of cell types, both pluripotent and multipotent stem cell types. Stem cell types that have been tested in stroke models are mesenchymal cells (MSCs)[145], embryonic stem (ES) cells[146], neural progenitors[147], and iPS cells[148-150].

A. Stem Cell Types

1. Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are a multipotent stem cell type and are an attractive cell type for stroke therapy because they can be harvested and transplanted autologously or allogeneically. MSCs are very clinically relevant since they are already being used clinically for transplantation for diseases such as leukemia, lymphoma, immunodeficiency disorders, and severe anemia. Autologous MSC transplantation has its benefits in circumventing immune rejection avoiding diseases such graft-versus-host disease (GVHD). This cell type, particularly bone mesenchymal stem cells (BMSCs) have been commonly studied in the laboratory for transplantation into stroke models[151,152]

and also been studied in clinical trials[153-155]. MSCs are multipotent and not pluripotent cells and thus have a limited neuronal differentiation potential. Whereas pluripotent cells can differentiate into any cell type in the body, MSCs are multipotent and their cell fate is generally committed to differentiating into stromal cells, osteoblasts, and chondrocytes. 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[156]. Nonetheless, this cell type has great value to the stroke field and has been shown to be therapeutic after stroke. Transplantation of BMSCs has reduced apoptosis infarct size in stroke models likely due to a trophic factor effect[152]. MSC transplantation has also increased functional recovery and cell proliferation[152,157].

In the clinical setting for stroke, MSCs have shown great promise. Some clinical trials using MSCs have demonstrated that their transplantation increases functional outcome in patients[154]. In one study, researchers aspirated stroke patients' MSCs and transplanted them autologously into the patient. The cells were isolated, cultivated and 50 million cells were transplanted at 5 weeks and again at 7 weeks after stroke. Compared to control patients, the MSC patient group had a better functional outcome according when followed-up 5 years after transplantation. Of note, there was also a correlation between outcome and the trophic factors such as SDF-1α measured from the patient serum 1 year after MSC treatment[154]. However, the group size for the study was too small to draw a conclusion about the effects of the elevated SDF-1α and the improvement in the MSC group.

2. Embryonic Stem Cells

Unlike MSCs, ES cells have the potential to differentiate into any cell type of the body since they are pluripotent. ES cells are harvested from the inner cell mass of a blastocyst. They are good candidates for cell replacement since they can differentiate down the neural lineage into any cell of the three germ layers and thus any neuronal cell type including glia, neurons, and oligodendrocytes. Numerous studies have tested ES cell transplantation into stroke animals and observed neuronal differentiation, regeneration, and an increase in functional recovery through behavior tests[147,158,159]. Nonetheless, these cells are controversial to use since they are derived from human embryos. Unlike MSCs, these transplantations would not be autologous since cells would have to be harvested from embryos. With allogeneic cell grafting, there is always the risk of immune rejection. Since ES cells are pluripotent cells there is also a risk for tumorigenesis. If cells are transplanted as pluripotent cells without differentiation down a certain lineage, they may become tumors without a differentiation direction. In most studies, pluripotent ES cells are pre-differentiated down a neural lineage into neural progenitors before transplantation[158,160,161]. They continue their differentiation into their terminal cell fate in the brain parenchyma[161,162].

Much work has demonstrated the capacity of ES cell-derived neural progenitors to differentiate into neurons in vivo, however more investigations are needed to understand the specific functioning and connections of these differentiated neurons. In other words, can transplanted cells interweave themselves into the endogenous circuitry to provide function? The ultimate stroke regeneration scenario would be for the cells to organize and mimic existing brain structures and networks.

Establishing cell transplantation circuitry is on forefront of this field. Some groups did address the cell connectivity after transplantation. Neural progenitors derived from primate ES cells were transplanted into the ischemic site and had terminally differentiated into neurons and glia cells after transplantation. In a study, a retrograde tracer, Fluorogold was injected into the anterior thalamus of an ischemic mouse brain to reveal that some grafted cells had taken up the tracer 28d after the transplantation in a different area indicating that transplanted cells do have the ability to form connections and synapse onto other brain structures after transplantation[161]. 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 emerging studies, we can begin to analyze the connectivity behaviors of the grafted cells and the cues that direct them to establish functional connections.

3. iPS Cells

iPS cells are also pluripotent cells that can be used in transplantation. iPS cells patient-specific cells are on the new frontier of personalized medicine where damaged tissue may be replaced with one's own derived cells[163,164]. In 2012, Yamanaka received the Nobel prize for his work in the creation of iPS cells. Yamanaka's group had first derived iPS cells from mouse fibroblasts in 2006, then later from human fibroblasts in 2007. iPS cells are adult somatic cells that undergo the upregulation of four genes that are required maintain pluripotency, Oct3/4, Sox2, Klf4, and c-myc and are genetically manipulated into pluripotent cells[148,149,165]. These cells are similar to ES cells in that they can differentiate into cell types of the three embryonic germ layers, but unlike ES cells,

they can be derived autologously from one's own somatic cells. For the purposes of transplanting into the brain, iPS cells can be differentiated down a neural lineage into neuronal progenitors and neurons[150,166]. Figure 3 shows that mouse iPS cell-derived neural progenitors can be differentiated into neurons in vivo after transplantation. These pluripotent cells can be also be differentiated into oligodendrocytes, astrocytes[167,168,169]. This technology is exciting to the field since one would be able to have their own stem cells generated from adult tissue. Since the cell transplantation would be autologous, the use of iPS cells would circumvent immune rejection issues as well as the ethical and political controversy surrounding the use of human embryonic cells.

A few groups have already studied the effects of transplanted iPS cells into stroke injury models. One of the first studies that used iPS cells transplanted them into a focal ischemic stroke model[150]. 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 and demonstrated the cells could differentiate into neurons with the expression of neurofilament and 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[150]. When testing functional recovery with the Rotarod and grasping strength after stroke and transplantation, animals with iPS cells and glue had the best functional recovery 1, 2 and 4 weeks after stroke induction[150]. This study used fibrin glue to provide a scaffold for transplanted cells which is a strategy to encourage cell survival and integration into the endogenous tissue.

Another study also demonstrated the beneficial effects of iPS cells[170]. This study differs from the previous study in that human iPS cells were transplanted and not mouse cells. Human iPS cells were generated from human fibroblasts and transplanted into rats with MCAO. iPS cells were derived from human fibroblasts in which Oct4, Sox2, Nanog, and Lin-28 were upregulated. Whereas Yamanaka's group could show reprogramming with Oct-4, Sox2, c-Myc, and Klf4[148], Thomson's group used a different set of factors, Oct4, Sox2, Nanog, and Lin-28[171]. Using the factors as Thomson did, this group was able to successfully generate human iPS cells. These cells were injected the cortex surrounding the infarct of rats that underwent a 70-minute 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[170]. 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[172]. The cells were differentiated into neuroepithelial-like stem cells and exhibited neuronal functionality via electrophysiology. iPS cell-transplanted mice showed a functional recovery after stroke through a staircase behavior test[172].

Even though these cells may seem like the ideal treatment for stroke injury since they can be derived and transplanted autologously and can differentiate into any cell type, there are still many optimizations and risks to be evaluated before translating this treatment into humans. For instance, iPS cells like any pluripotent cell type have the possibility for tumorigenesis after transplantation. Also, the proper dosage of cell delivery, age of cells used, and timing must be optimized. The generation of iPS cells from one's self will take time and need to be optimized if they are to be transplanted at a

target time after a stroke. Even though has shown iPS cells improving symptoms in models ischemic stroke, cell survival, chemoattractive mechanisms, and the trophic factor benefits in iPS cell transplantation have not been explored in stroke[159,166].

B. Routes of Transplantation

1. Intracerebral Transplantation

The advantages to transplanting via the intracerebral method is that it is direct and the location of the transplantation can be specified. The drawbacks are that these transplantations can be invasive requiring a craniotomy surgery to deliver the cells locally to the brain tissue. Instruments to inject the cells such as a needle or pipette have the potential to damage the surrounding brain parenchyma. Transplanting into a suitable microenvironment for stem cells allows for increased survival of the graft. For example, direct transplantation into the stroke core exposes the transplanted stem cells into the harsh environment of cytotoxicity and inflammation. Often a strategy for graft survival is transplanting the cells into the penumbra avoiding the core of the stroke. Transplantations into the core have lower survival rates compared to transplantations into the penumbra[173,174]. The penumbra provides a more favorable microenvironment for transplanted cells[147,158,175]. There are other methods to deliver cells, however each come with advantages and drawbacks.

2. Intravascular Transplantation

Stem cells may also be delivered through the vasculature either intra-arterially or intravenously. This method is less invasive than intracranial transplantation and does not necessitate a craniotomy, however the drawback is that cells must home to the injury to

provide a benefit. Vascular administration has demonstrated various forms of functional improvement in stroke animals. Intravenous administration of cord blood stem cells in rat stroke animals showed an improvement in motor functional recovery when tested with the Rota-rod test[176]. In similar studies, the intra-arterial administration of MSCs improved the Neurological Severity Score *(*NSS) score and performance in the adhesive-removal test in stroke rats[177].

There are differences between intravenous and intra-arterial vascular delivery. The cells delivered intravenously circulate throughout the body and pulmonary circulation where the cells might get entrapped in other organs (mainly the spleen, liver, and lungs)[178,179] and consequently fewer cells localize to the brain. In contrast, cells delivered intra-arterially circumvent the systemic circulation and more cells enter the brain. In one study, 21% of intra-arterially delivered cells compared to 4% with intravenous administration entered the brain[177]. Similarly, another study reports that 1300 cells/mm^2 entered the infarct area after intra-arterial administration compared to 74 cells/mm^2 after intracarotid administration.

Much of cell homing relies on trophic and chemoattractive factors in the tissue to provide localization cues. For example, the delivery of trophic factors with the cells increases the proportion of administered cells that reach the stroke site. Studies demonstrated that the intravascular injection of BMSCs with subsequent injections of granulocyte-colony stimulating factor (G-CSF) and stem cell factor (SCF) increased BMSC migration in the circulation and cell homing to the brain[180,181]. Furthermore, others have also demonstrated that BSMCs entered the ischemic brain two weeks after stroke

and treatment with BMSCs, G-CSF and CSF suggesting that vascular BMSC therapy supplemented with trophic factors can be beneficial after stroke.

3. Intranasal Administration

Intranasal adminstration (INA) of stem cells offers two main advantages that are important for the treatment of ischemic stroke. The advantage of INA is the ability for cells to non-invasively access the CNS from the nasal mucosa. However unlike intracerebral transplantation, not all cells will localize to the infarct. Though the route of migration is not fully understood, it is thought that transplanted cells bypass the BBB and move from the nasal mucosa into the CNS through the cribriform plate migrating into the brain parenchyma along the olfactory neural pathways, the corpus callosum, and blood vessels. Studies have demonstrated that through INA delivery, MSCs can enter several areas of the brain parenchyma including cortex, hippocampus, striatum, cerebellum, and brainstem[152,182,183]. INA transplantation of MSCs have been demonstrated to be effective in stroke models. MSCs can migrate through the intact brain after intranasal delivery rapidly reach the brain only 1 hour after intranasal delivery through either the rostral migratory stream or the cerebrospinal fluid[183]. INA was also tested in neonatal stroke models and was found that MSCs remained in the brain 28 days after delivery and had also improved sensorimotor functional recovery[184].

C. Summary

Stem cell transplantation into the stroke brain holds great promise for tissue regeneration. “Stem cells” encompasses a broad category of cells. Those tested in a

stroke model can be multipotent, or pluripotent. They also different in the source of the cell; some may be autologously harvested such as MSCs, others may be from an embryonic source, or derived from one's adult somatic cells. The modes of transplantation/ administration vary and the researcher must weigh the benefits of direct access to the stroke tissue and greater cell localization with procedure invasiveness of the transplantation and fewer homing of the cells. The logistics of cell dosage, timing, and production/harvesting of autologous cells must be further characterized and resolved in preclinical studies before translation.

Chapter IV

Stem Cells and Trophic Factors

Stem cell transplantation may act in two beneficial ways: 1. Provide cell replacement to dead or dying tissue, 2. Encourage several aspects of regeneration by the release of trophic factors to the injury site. Several groups have been successful in demonstrating that transplanted cells such as ES[158,185] or iPS[158,172] cells have differentiated into neurons. At this point, few studies have observed functional long-distance connections from transplanted differentiated cells[161], yet the stroke brain still benefits from stem cell trophic factor support after transplantation even if neuronal differentiation or long-distance connections do not occur.

Transplanted stem cells exhibit paracrine effects to the surrounding tissue. In some cases, the lack of or withdrawal of a trophic factor such as nerve growth factor (NGF) can trigger cell death[186]. Stem cells naturally express factors including vascular endothelial growth factor (VEGF), fibroblast growth (FGF), BDNF, and EPO that encourage repair. The idea that stem cells are effective vehicles and secretors of trophic factors is further supported with studies that applied BMSC-conditioned media into a stroke brain led to functional benefits. BMSC-conditioned media can recapitulate some effects of the cell transplantation itself. BMSC-conditioned media has been reported to increase neurite outgrowth, increasing neurite length and branch number in Ntera-2 neurons supporting BMSC paracrine effects[187]. MSCs release a wide range of adaptive factors including factors that are cytoprotective (endothelin), angiogenic (VEGF, Smad4, Smad7), and involved in cell migration (LRP-1, LRP-6)[188]. In a human study of stroke patients, BMSCs were harvested autologously from each patient and transplanted at several points at the perilesional area. Neurotrophin levels like BDNF and NGF were

significantly increased in the brain tissue after BMSC transplantation as detected with ELISA demonstrating that stem cell grafts are trophic factor vehicles[189].

A. Trophic Factors From Stem Cells Promote Regeneration

One of the major events of tissue regeneration involves rebuilding the neurovascular unit after stroke. The neurovascular unit is a functional unit in the brain where the neurons, astrocytes, and pericytes of the CNS interface with the circulatory system to maintain metabolic homeostasis such as ionic concentrations. This dynamic regulatory unit is compromised after an ischemic event, however this unit may be repaired by angiogensis and neurogenesis. Previous work has demonstrated that angiogenesis and neurogenesis are causally linked to regenerate the neurovascular unit[80]. When angiogenesis was blocked with Endostatin after stroke, there was a correlating decrease of new neuron formation[80]. Neurogenesis and angiogenesis synergistically regenerate functional tissue after stroke.

1. Trophic Factors From Stem Cells Promote Angiogenesis

In order for stem cell transplantation to improve upon a stroke injury, several reparative events must take place including rebuilding the vasculature[80]. A major trophic factor is VEGF for the stimulation of angiogenesis. In vitro studies demonstrate that tubule formation of endothelial cells (human umbilical vein endothelial cells, HUVEC) are stimulated when VEGF is present resulting in increased vessels[190]. The effect is attenuated with the addition of VEGF antibody[190]. In vivo, VEGF secreted from transplanted neural stem cells increased neovascularization and the attenuation of

inflammation in the peri-infarct area[191]. In animals that received neural stem cell transplantation in the peri-infart area, there was a greater blood vessel density compared to naïve and controls at two weeks post-transplantation. Further, animals with neural stem cell transplantation displayed enhanced angiogenic signaling pathways exhibiting greater levels of pVEGR2, Tie-2, both receptors for ligands VEGF and Angiopoietin 1 and 2. In a separate study, neuroepithelial-like stem cells derived from human iPS cells express VEGF[172]. Transplantation into the stroke mouse brain also shows an increase of VEGF in the astrocytes and blood vessels in that area surrounding the graft[172]. Interestingly, this study was not able to show increased angiogenesis due to the released VEGF suggesting that the VEGF released played a role in the improved functional recovery observed, or perhaps there was not enough VEGF secreted to cause an angiogenic effect. SDF-1α is also contributes to angiogenesis after stroke by attracting of endothelial cells for revascularization[192,193]. SDF-1α and VEGF both play roles in endogenous neural progenitor migration as well as endothelial progenitor cell recruitment[132,194]. Endogenous endothelial cell progenitors express CXCR4 and are chemoattracted to SDF-1α and VEGF[116,195]. In all the contribution of trophic factors from and stem cells to angiogenesis after the stroke site can be measured and evaluated several ways, through blood vessel density, Western blot of angiogenic receptors, through functional recovery, local cerebral blood flow[196], or vessel and BrdU co-label in immunohistochemistry (Figure 4.1).

2. Trophic Factors From Stem Cells Promote Neuroprotection and Neurogenesis

Stem cells can enhance neurogenesis and neural progenitor migration via trophic factor secretion of SDF-1α, VEGF, and BDNF. Aforementioned, chemoattractive trophic

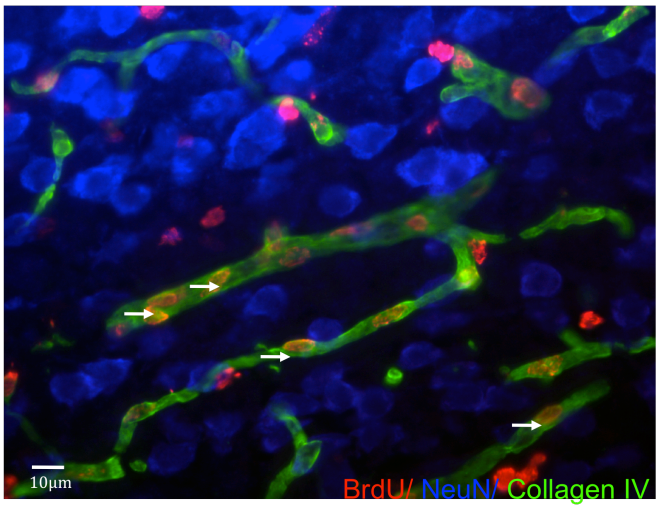


Figure 4.1 Immunohistochemical method of measuring angiogenesis. Stem cell transplantation increases angiogenesis. Since BrdU is a marker of proliferating cells, angiogenesis can be quantified by counting the number of Brdu (red) co-labeled with basement membrane marker (Collagen IV, green). Several co-labeled cells can be observed here (white arrow)

factors like SDF-1α play a crucial role in homing endogenous neural progenitors to the stroke lesion[82,132]. Since SDF-1α has already been thoroughly discussed in Chapter 2 of this dissertation, this section will focus on other major neurotrophic factors.

VEGF has mainly been studied in angiogenesis, however VEGF plays a role as a neuroprotective and neurogenic factor as well. Mice over-expressing VEGF had fewer neurological deficits and smaller infarct volumes after stroke compared to mice without over-expression[197]. Trophic factors can prevent cell death through its 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 the mediation of VEGFR-1, one of the VEGF receptors[198]. VEGF has also been shown to promote neurogenesis in the SVZ and SGZ and endogenous migration of neural progenitors from the SVZ[199]. Thus, VEGF has neurogenic, neuroprotective, and angiogenic abilities. BDNF is another trophic factor released by stem cells have been shown to provide neurotrophic properties and play a role in neurogenesis[200,201]. BDNF intersects with apoptotic pathways to prevent cell death as seen in cerebellar neurons treated with BDNF[162]. There was more survival with granule neurons treated with 50ng/ml of BDNF

compared to cells with no treatment[162]. These neurotrophic factors are often pleiotropic and play a role in neurogenesis.

B. Strategies to Enhance Trophic Factor Expression

1. Genetic Upregulation of Trophic Factors

In the pursuit to enhance the beneficial aspects of stem cells, one strategy is to genetically engineering stem cells to enhance their trophic factor release (Table 2). With the current plethora of plasmid cloning tools available, this approach to trophic factor upregulation has become more and more feasible. Many plasmid backbones are pre-constructed with a promoter, fluorescent reporter genes like GFP, and mammalian and bacterial selection markers. These plasmids are accessible via global plasmid repositories such as Addgene or subcloning kits.

Several groups have shown that the upregulation of trophic factors in transplanted cells can improve stroke outcome even more than cells without the upregulation. Neonatal rats with ischemic stroke received intranasal delivery of MSCs with upregulated BDNF[151]. The BDNF gene was cloned into the pShuttle2 vector under the CMV promoter and delivered via adenoviral vector transduction. Overall, MSC and MSC-BDNF transplantation both showed positive effects upon the stroke injury, but some outcomes showed greater improvement with BDNF upregulation in the cells. Even though infarct size and gray matter loss did not show a difference between MSCs and MSCs with BDNF, there were some outcomes that did show a difference with BDNF upregulation. Rats transplanted with MSC-BDNF cells had an improved motor abilities at 4d after stroke compared to MSC cells only[151]. Placental growth factor has also been upregulated in MSCs[202]. When transplanted into the brain after stroke, MSCs with PlGF consistenly

showed a smaller lesion volume measured with MRI for 24h to 7d compared to MSCs without PlGF[202]. Sandwich ELISA assay indicates an in vivo production of PlGF after transplantation at 3 and 7d[202]. Also PlGF reduced cell death (measured by TUNEL) at the ischemic boundary of animals transplanted with MSCs with PlGF upregulation compared to animals with MSCs without PlGF upregulation[202].

Upregulating an anti-apoptotic factor to reduce apoptosis has been shown to be beneficial as well. Wei and colleagues engineered ES cells to upregulate the anti-apoptotic factor, Bcl-2[158]. Bcl-2-upregulated cells showed better survival than cells without upregulation in vivo[158]. Transplantation of the Bcl-2-upregulated cells improved functional recovery in animals with transient cerebral ischemia[158]. Bcl-2 is an anti-apoptotic factor in the intrinsic apoptosis pathway and the study showed that the upregulation of this factor does indeed lead to greater cell survival in vivo[158]. Promoting cell transplant survival through the upregulation of survival factors is an emerging method in the field of stem cell transplantation for stroke therapy. These reported studies not only demonstrate that cell transplantation itself can be beneficial in supporting regeneration and functional recovery, but that the upregulation of beneficial factors through genetic upregulation can further enhance the regenerative actions of the transplant.

Factor Upregulated	Stem Cell Type	Upregulation Method	Citation
BDNF	mMSC	pShuttle2 vector, CMV promoter, adenovirus infection	Mesenchymal stem cell transplantation attenuates brain injury after neonatal stroke. van Velthoven, C. T. et al. Stroke **44**, 1426-1432 (2013)[151]
Placental Growth Factor (PlGF)	hMSC	fibre-mutant F/RGD adenovirus vector, adenovirus infection	Neuroprotection by PlGF gene-modified human mesenchymal stem cells after cerebral ischaemia. Liu, H et al. Brain **129**, 2734-2745 (2006)[202]
Ang-1	hMSC	pCAcc vector, CAG promoter, adenovirus infection	Therapeutic benefits by human mesenchymal stem cells (hMSCs) and Ang-1 gene-modified hMSCs after cerebral ischemia. Onda, T et al. J Cereb Blood Flow Metab **28**, 329-340 (2008)[203]
GDNF, CTNF, NT3, BDNF	hMSC	fiber-mutant adenovirus vector, CA promoter, adenovirus infection	Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model. Kurozumi, K. et al. Molecular therapy : the journal of the American Society of Gene Therapy **11**, 96-104 (2005)[204]
Bcl-2	mESC	pcDNA3-based plasmid, CMV promoter, transfected via electroporation	Transplantation of embryonic stem cells overexpressing Bcl-2 promotes functional recovery after transient cerebral ischemia. Wei, L et al. Neurobiol Dis **19**, 183-193 (2005)[158]

Table 4.1. Genetic modifications of stem cells transplanted into stroke models. Several groups have genetically upregulated trophic factors or survival factors as a strategy to enhance cell survival after transplantation or to use the cells as a vehicle for delivering a trophic factor.

2. Hypoxic Preconditioning

Other methods to upregulate trophic factors in stem cells take advantage of endogenous hypoxia regulatory systems. The use of hypoxic preconditioning has been a clever strategy to bolster cell survivability and a way to increase trophic factor expression. One of the major obstacles of stem cell transplantation into the stroke brain is that a large proportion of the transplanted cells die[205]. Exogenous cell death may be due to an inhospitable host environment that may be cytotoxic from the acute phase of ischemic stroke or due to the mechanical injury of the injection or during harvesting of the cells for transplantation[83]. Cell transplantation must be appropriately timed so that the transplantation is not too early and encounter cytotoxic substrates associated with acute ischemia.

a. HIF-1α Mechanism

Preconditioning is the phenomenon in which delivering a sub-lethal stimulus to an organism primes it for a greater subsequent insult. Ischemic and hypoxic preconditioning have been used in animals and in cell culture models to increase the organism's tolerance a subsequent insult. One of the first studies of ischemic preconditioning was reported by Dahl, et al in 1964[206]. They showed that exposing a rat to a brief period anoxia allowed the rat to tolerate and survive a prolonged anoxic exposure[206]. Ischemic and hypoxic preconditioning primes the system through HIF-1α.

The HIF-1α system is a major regulator of oxygen homeostasis in the cell bringing about 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 hydrolyate 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 allowing HIF-1α to heterodimerize with HIF-1β and translocate into the nucleus where it will bind to hypoxia response elements (HREs) in the targeted genes. Adaptive genes like those for VEGF, erythropoietin (EPO), BDNF, glial cell line-derived neurotrophic factor (GDNF), and SDF-1α are upregulated[207].

This preconditioning paradigm has been used in in vitro models of cell cultures to bolster their survivability for transplantation[147,152,208]. Our group's investigations show that hypoxic preconditioning of neurally-differentiating mouse embryonic stem cells for 12h under 0.1% oxygen 24h before exposing them to serum deprivation revealed less cell death; fewer terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells, caspase-3 cells, and less lactate dehydrogenase (LDH) release was reported[52]. Hypoxic preconditioning of cells results an upregulation of Bcl-2 and fewer indicators of cell death[147]. The priming under 0.1% oxygen hypoxic conditions enhanced the cells' endogenous protective mechanisms to allow tolerance to the subsequent insult of serum deprivation. Similarly, with human ES cells differentiated into neural progenitors, hypoxic preconditioning for 12h at 0.1% oxygen upregulated HIF-1α, VEGF, EPO and Bcl-2[208]. These preconditioned neural progenitors were also more likely

to differentiate into neurons compared to cells that had not been preconditioned[208]. The implications for using preconditioning before transplantation are great due to the neural regenerative potential.

BMSCs have also showed a similar response to preconditioning. In a focal ischemic stroke model, INA of BMSCs that were preconditioned showed an increase in CXCR4 expression, MMP-2, MMP-9, and cell survival after preconditioning[152]. The preconditioning of transplanted cells improved outcomes in stroke mice due to the increased migratory capabilities of cells via CXCR4 upregulation. Animals with preconditioned cells also showed greater behavioral recovery at 14d after stroke compared to those that received cells without preconditioning[152].

C. Summary

Transplantation of different types of stem cells into stroke models have shown to encourage neurogenesis, neuroprotection and angiogenesis. Using stem cells as vehicles for trophic factor delivery is as important as their ability to replace lost cells. Stem cell transplantation, especially with iPS cells is on the forefront of treatment research showing anti-apoptotic factor expression, reduced infarct size, and increased functional recovery in vivo. Even though cell transplantation has several therapeutic effects, it still requires optimization. There are several strategies to promote cell transplant survival through the upregulation of trophic factors. One way to increase such factors is to upregulate trophic factor with targeted genetic manipulation, method that is of growing importance in basic research. Another method to maximize cell survivability for transplantation is hypoxic

preconditioning to increase trophic factor expression and cell survivability through the HIF-1α mechanism.

Chapter V

Rationale, Aims, Experimental Methods

A. Rationale and Significance

Ischemic stroke is a cardiovascular disease that affects a staggering number of people worldwide. Many clinical trials have been unsuccessful in demonstrating that the neuroprotective agents can effectively treat stroke[16]. Thrombolytic agents such as tPA have been efficacious, but its administration is limited to a small timeframe after stroke. Stem cell transplantation has been shown to be beneficial after stroke due to their abilities to replace damaged cells and their paracrine trophic effects, however depending on the political climate and ES cell accessibility, ES cells may not be a sustainable source of pluripotent cells for transplantation. Thus, iPS cells provide a suitable patient-specific alternative for cell transplantation after stroke.

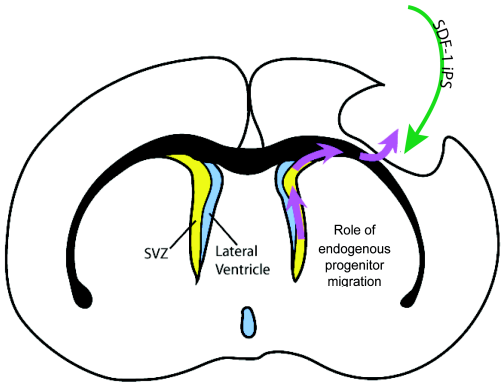


Figure 5.1. Schematic of iPSC Transplantation. The SVZ is a source of proliferating neural progenitors that migrate to ischemic areas. SDF-1 directs migration from the SVZ to the infarct. Our objective is to enhance progenitor migration by transplanting iPS cells with SDF-1 upregulation to for improved stroke repair

A great percentage of cells die upon transplantation into the stroke brain. Thus, there is a need to bolster cell survival before transplantation. Similarly, many endogenous neural progenitors die upon reaching the stroke injury likely due to the cytotoxic nature of the injury[83]. One strategy is to increase both cell survival and to further chemoattract endogenous neural progenitors to the infarct by upregulating SDF-1 in transplanted cells. SDF-1 increases cell survival and increases endogenous cell chemoattraction[132]. This investigation will be an original effort to use iPS cells in a cell-based therapy for ischemic stroke. The novel approach of genetically modifying iPS cells with a pro-survival/pro-migration gene will likely demonstrate a

beneficial strategy of combining enhanced exogenous and endogenous stem cells for a regenerative stroke treatment.

B. Specific Aims

Aim 1- To test the regenerative abilities of iPS cell-derived neural progenitors (iPSC-NPCs) in a neonatal model of ischemic stroke

Hypothesis- Transplanting iPSCs differentiated into iPSC-NPCs will increase regenerative activities in a model of focal ischemic stroke

1.1 To test the differentiation capacities of iPSC-NPC in vitro and after transplantation

1.2 To test trophic factor expression in iPS-NPCs in vitro and after transplantation

1.3 To test angiogenesis and neurogenesis after transplantation

1.4. To test the functional recovery after transplantation

Aim 2- To determine the role of SDF-1α upregulation in iPS cell survival and neuronal differentiation in vitro

Hypothesis: SDF-1α upregulation in iPS cells will enhance survival and neural differentiation in vitro.

2.1 To test the survival of iPS cells with SDF-1α upregulation in an in vitro model of ischemia (oxygen-glucose deprivation, or OGD)

2.2 To test the neuronal differentiation of iPS cells with SDF-1α upregulation

2.3 To confirm the functionality of iPS-cell derived neurons with SDF-1α upregulation

Aim 3- To investigate the chemoattractive role of SDF-1α in iPSC-NPCs after transplantation

Hypothesis: Transplantation of SDF-1α-upregulated iPSC-NPCs into the stroke peri-infarct area will increase endogenous progenitor migration and functional recovery

3.1 To determine the ability of SDF-1α-upregulated iPSC-NPCs to enhance endogenous progenitor migration in vivo

3.2 To test the functional recovery of mice with stroke and SDF-1α-upregulated iPSC-NPC transplantation

C. Materials and Methods

iPSC Culture and Differentiation

Pluripotent mouse primary iPSCs (WP5 line) were purchased from Stemgent (Cambridge, MA) and maintained in their pluripotent state in a growth culture medium consisting of 15% ES cell fetal bovine serum (ES Cell FBS, Gibco Life Technologies, Grand Island, NY) in Dulbecco's Modified Eagle Medium (DMEM; Corning Cellgro, Manassas, VA). This media was supplemented with 1% non-essential amino acids, 1% penicillin-streptomycin, 0.1% beta-mercaptoethanol, 1:10,000 leukemia inhibitory factor (LIF). Cells were either passaged daily with 0.25% trypsin-EDTA when cells were approximately 80% confluent or had their media changed if the cells were not confluent enough to passage. Pluripotent cell culture was maintained on mouse embryonic fibroblast (MEF) feeder layers (Millipore, Billerica, MA). iPSCs were differentiated in suspension culture with a "4-/4+" (4 days without/4 days with) retinoic acid (all trans-retinoic acid, RA, Sigma, St. Louis, MO) protocol in LIF-free media[169,209]. Cells were

dissociated from the flask with 0.25% trypsin without EDTA. During dissociation, the MEFs were allowed to adhere to the dish allowing the floating iPSCs to be isolated from the MEFs. Four million dissociated iPSCs were plated in petri dishes for suspension culture in growth media without LIF. Within the first day, the cells formed embryoid bodies in suspension. In the last four days, 5 x 10^{-7} M of all-trans RA was added to the media. Neural progenitors were harvested by dissociating the cells with 0.25% trypsin-EDTA for 10 min at 37°C, inactivated with serum media, and run through a cheesecloth filter to separate the cells. The cells were centrifuged and resuspended to a concentration of 100,000 cells/μl and were either transplanted immediately into the stroke rats or plated in modified SATO media[210] on Poly-D-Lysine (PDL)/ laminin-coated dishes and allowed to terminally differentiate for 5 days (Figure 1).

SDF-1α Upregulation Plasmids

Human elongation factor 1α (EF1α), Chicken β-actin (CAG), and cytomegalovirus (CMV) promoter plasmids were constructed and subcloned by Oskar Laur at the Emory University Vector Core. The EF1α plasmids were created from the backbone, pEGIP obtained from Addgene (plasmid # 26777). The plasmid backbone (pEGIP) contained the EF1α promoter, GFP reporter gene, puromycin resistance for selection, and an internal ribosome entry site (IRES) expression system. The EF1α-GFP-SDF-1α plasmid was created with the 2A expression system. GFP and SDF-1α were both subcloned in under the EF1α promoter. The empty plasmid and the SDF-1α plasmid were packaged into lentiviruses (see below).

Lentivirus Production and Stable Cell Line Creation

The plasmids were packaged into lentiviruses by Xinping Huang at the Emory University Viral Core. HEK 293FT (Invitrogen) cells were maintained in medium (4.5g/L Glucose and L- Glutamine containing DMEM supplemented with 10% FBS and 1% Pen-Strep) and incubated at 37^0C, 5% CO_2. One day before transfection, HEK 293FT cells were seeded onto ten 150mm plates at a density of $1x10^7$ cells per plate in 20 ml of complete medium. The cells were approximately 70-80% confluent at the time of transfection. On the day of transfection, the following mixture was prepared: 250µg of FDS1 or control plasmid + 187.5µg of pCMVdelta 8.9 + 75µg of pV-SVG + 12ml of ddH_2O + 12.5ml of 0.5M Ca_2Cl + 25 ml of 2x HeBS to total volume 50ml, this solution was vortexed for a few seconds and incubated for 20min at room temperature, and then 5ml of the mixture added dropwise to the each dish. The dishes were returned to the incubator. 7 hours post-transfection, the medium was replaced with 20 ml of fresh medium and incubated for an additional 48h before harvesting. The supernatant containing the lentivirus were collected 2 days after the 48h and 72h post-transfection, 2 days supernatant were combined and was centrifuged at 500xg for 5min at 4^0C, followed by passage through a 0.45µm low protein binding filter. The total 400ml of supernatant was loaded to six 70ml ultracentrifuge tubes in centrifuged at 28,000rpm for 2h at 4^0C in a 45Ti rotor (Beckman). The virus pellets were resuspended in 500µl of PBS, incubated on ice for 30min, the six tubes of resuspended virus were combined and then loaded to a 12ml SW 41 tube. 3ml of 20% sucrose was added as a cushion then ifuged at 28,000rpm for 2h at 4^0C in a SW 41 rotor (Beckman). The virus pellet was resuspended in 100µl of PBS and incubated for 2h at 4^0C. This was then aliquoted and stored at -80^0C.

5μl of virus was applied to iPS cells maintained in 3.5cm dish so that can stay in dish with virus for 1d without passaging. One day after application, GFP was confirmed in control and SDF-1 cells. The cells were trysinized and passaged into T25, or 25cm^2 flasks. Viral titers were 1x10^8 IU/ml for pEF1α-EGFP and 1x10^7 IU/ml for pEF1α-EGFP-2A-SDF. Pluripotent cells were infected with the control and SDF-1 viruses. To create stable cell lines of iPSCs expressing GFP and GFP-SDF-1α, we utilized the puromycin (10mg/ml) selection in the plasmid backbones. Cells were allowed to proliferate for 2 days before adding puromycin. The cells were allowed this time to express the puromycin resistance. Puromycin was initially added at 1:500 concentration for initial selection, then maintained at 1:10,000 concentration.

Plasmid Transfection

Lipid transfection through Lipofectamine 2000 was used to test plasmid upregulation initially. Mouse iPS cells were plated in 24-well plates in media (500μl/well) without antibiotic and transfected when cells were 90% confluent. For each transfection sample, 1μg of DNA plasmid was incubated with 50μl of Opti-MEM. 1μl of Lipofectamine 2000 was incubated with 50μl of Opti-MEM for 5min. The two mixtures are combined into a 100μl mixture to incubate for 25min at room temperature. All of the mixture is applied to the cell media and mixed by rocking back and forth. The media (no antibiotic) is changed after 4-6h.

Oxygen Glucose Deprivation

As an in vitro model of ischemic stroke, oxygen glucose deprivation (OGD) was performed on the control and SDF-1-upregulated cells. iPS cells were plated into 24-well plates at 200,000 cell/ well. After adhesion to the bottom of the well with overnight incubation overnight, cells were incubated for 3-7 hours in the ProOx-C-chamber system (Biospherix, Redfield, NY) at 0.1-0.3% oxygen at 37°C. Control cells were maintained in normoxic conditions. After preconditioning, the cells were returned to normoxic, culture conditions for a 12 hour reoxygenation period. Following reoxygenation, cultures were assayed with the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

MTT Assay

25 µl of MTT solution (5mg/mL) was added to 250 µl of cell media per well and incubated for 4 hours at 37°C. 250 µl of solubilization solution was added to each well and incubated overnight. The solubilization solution consists of 10% sodium dodecyl sulfate (SDS) in 0.01M HCl. Wells were read on a plate reader at 560nm to measure optical intensity.

Enzyme-Linked Immunosorbent Assay (ELISA)

Pluripotent cells in maintenance culture were plated into the wells of a 6-well plate at 1.5 million cells/ well such that the cultures were 80% confluent the day after plating. On the day of experimentation, the cells' media was changed with the addition of 1ml of normal FBS growth media. The media was incubated with the control and SDF-

1α cells for 8h before media collection. After 8h, all of the media were collected separately from each well and cell debris was spun down via centrifugation. 100µl from each sample was used for assaying using an ELISA for human SDF-1α (Raybiotech, Norcross, GA).

Samples and SDF-1 standard protein were incubated in duplicate on the ELISA plate sealed and shaking overnight at 4°C. After incubation, the solutions were discarded from each well and rinsed with 300µl wash solution 4 times with a multi-channel pipette. Biotinylated SDF-1 antibody was added to each standard and sample well, incubated for 1h at room temperature with shaking. The solution was discarded then washed with wash solution 4 times. Strepavadin solution was prepared and applied to the wells for 45 min of incubation with shaking. The solution was discarded then washed with wash solution 4 times. TMB One-Step Substrate Reagent was applied and incubated for 30 min. Stop solution was applied to halt the reaction and the plate was read at 450nm on a plate reader. The duplicate values were averaged and calculated against the standard curve reading.

Immunocytochemistry

Cell cultures were fixed with 4% paraformaldehyde for 15 min. Cells were washed 3 times for 5 min each with phosphate-buffered saline (PBS) subsequent to each protocol step. The cells were treated with -20°C ethanol/acetic acid solution. Cells were permeabilized with a 0.2% Triton-X 100 solution, and blocked with 1% cold fish gelatin (Sigma, St. Louis, MO). Cells were incubated overnight at 4°C with primary antibodies for Oct3/4 (sc-5279, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA), Sox2 (sc-17320,

1:200; Santa Cruz Biotechnology), doublecortin (DCX, sc-8066, 1:100; Santa Cruz Biotechnology), NeuN (MAB377 1:200; Millipore), β3 Tubulin (Tuj-1, MMS-435P, 1:200; Covance, Princeton, NJ), synaptosomal-associated protein 25 (SNAP-25, AB5871P, 1:100; Millipore), synapsin 1 (51-5200, 1:200; Life Technologies, Grand Island, NY), neurofilament (AB9568, 1:100; Millipore). To analyze trophic factors, iPSC-derived neural progenitors (iPSC-NPCs) were incubated overnight with primary antibodies at 1:100 for FGF (sc-7375; Santa Cruz Biotechnology), BDNF (sc-546; Santa Cruz Biotechnology), EPO (sc-1310; Santa Cruz Biotechnology), SDF-1α (MAB350; R&D systems, Minneapolis, MN), GDNF (sc-328; Santa Cruz Biotechnology), and GFP. After incubation overnight, cells were washed with PBS 3 times for 5 min each. Secondary antibodies were applied at 1:100 for 1 hr at room temperature for Oct3/4 and SDF-1α, NeuN, Tuj-1 (donkey anti-mouse Cy3, Jackson ImmunoResearch, West Grove, PA), DCX (donkey anti-goat Cy3, Jackson ImmunoResearch), GDNF (donkey anti-rabbit Cy5, Jackson ImmunoResearch), Sox2, FGF, EPO, SNAP-25 (AlexaFluor 488, donkey anti-goat; Invitrogen Life Technologies, Grand Island, NY), and BDNF, neurofilament, synapsin 1 (donkey anti-rabbit Cy3, Jackson ImmunoResearch). Dishes were washed with PBS and stained with Hoechst 33342 for 5 min (1:20,000), washed, and cover-slipped with Vectashield mounting media (Vector Laboratories, Burlingame, CA).

Electrophysiological Characterization

Whole-cell patch clamp recording was performed on mouse iPSC-derived neurons at 10 days after neural progenitor harvest using an EPC9 amplifier (HEKA, Elektronik, Lambrecht, Germany) at room temperature. The recording external solution

contained (mM) 135 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 Glucose at a pH of 7.4. Recording electrodes were pulled from borosilicate glass pipettes (Sutter Instrument, USA) had a tip resistance between 5 and 8 MΩ when filled with the internal solution (mM): 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 2 Na_2ATP, 10 ethylene glycol tetraacetic acid (EGTA), and 10 HEPES at a pH of 7.2. Series resistance was compensated by 60-80%. Linear leak and residual capacitance currents were subtracted on-line using a P/6 protocol. Action potentials (APs) were recorded under current-clamp mode using Pulse software (HEKA, Elektronik). Tetrodotoxin (TTX, 1μM) was used to block voltage-gated inward sodium currents. Data were filtered at 3 KHz and digitized at sampling rates of 20 KHz. AP amplitude was determined by measurement from the initial threshold to the peak of AP upstroke. All the electrophysiological data in this study are expressed as mean ± SD.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) on iPSC-NPCs

On the final day of the differentiation protocol (day 8) when the iPSCs had differentiated into neural progenitors, the total mRNA was extracted from the cells with Trizol reagent (Invitrogen Life Technologies). For each dish, 250 μl of Trizol was used to lyse the cells. 50 μl of choloroform was added and samples were centrifuged. The RNA was collected from the upper aqueous phase and precipitated out with 125 μl of isopropyl alcohol. The RNA was centrifuged down into a pellet and washed two times with 75% ethanol. The RNA pellet was air-dried and resuspended in diethylpyrocarbonate (DEPC)-treated water and the RNA concentration was measured. The High Capacity RNA-to-cDNA kit™ (Applied Biosystems Life technologies, Grand Island, NY) was used to

create cDNA from the RNA samples. PCR reactions are a mixture of Taq buffer (New England Biolabs, Ipswich, MA), forward primer, reverse primer, dNTP (10mM), Taq polymerase, water and cDNA. Primer pairs for trophic factors (mouse) were designed from the mRNA sequence in the NCBI nucleotide bank. The primer pairs are listed below. DNA samples were run out in a 1.8% agarose gel and band intensity was quantified using ImageJ software (NIH, Bethesda, MD).

Primer pairs:

18S: GACTCAACACGGGAAACCTC (forward), ATGCCAGAGTCTCGTTCGTT (reverse)

BDNF: CGACATCACTGGCTGACACT (forward), ATGTTTGCGGCATCCAGGTA (reverse)

CXCR4: GCCATGGCTGACTGGTACTT (forward), CACCCACATAGACGGCCTTT (reverse)

EPO: ACCACCCCACCTGCTCCACTC (forward), GTTCGTCGGTCCACCACGGT (reverse)

FGF1: GTGGATGGGACAAGGGACAG (forward), GGTGTCTGCGAGCCGTATAA (reverse)

GDNF: CGCTGACCAGTGACTCCAAT (forward), CTCTGCGACCTTTCCCTCTG (reverse)

SDF-1α: GCTCTGCATCAGTGACGGTA (forward), CCAGGTACTCTTGGATCCAC (reverse)

VEGF: CTCACCAAAGCCAGCACATA (forward), AAATGCTTTCTCCGCTCTGA (reverse)

Focal Ischemia Surgery and iPSC-NPC Transplantation

Adult mice (2-3 month-old) or Wistar rat pups and dam were housed in a climate-controlled room with a 12-hour light-dark cycle. Animals had free access to water and food. Rat pups received experimental treatment from age P7 to P28 and were sacrificed by 4% chloral hydrate (10 ml/kg) anesthesia before decapitation. Mice received experimental treatment at 2-3 months of age and were also sacrificed by 4% chloral hydrate (10 ml/kg) anesthesia before decapitation. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University.

Rats:

To induce focal cerebral ischemia, P7 rat pups were anesthetized with hypothermia as described[211]. Surgery commenced when the rat showed no response to pinches to test reflexes. The right common carotid artery (CCA) and the distal branches of right middle cerebral artery (MCA) were permanently occluded via cauterization to produce an ischemia in the right whisker barrel cortex. Sham animals received skin incisions, but no arterial occlusions. Starting 3 days after stroke, animals received a daily intraperitoneal (i.p.) injection of Bromodeoxyuridine (BrdU; Sigma) to label any proliferating cells in the brain. Pups were given 1/3 the adult BrdU dosage (16.7 mg/kg) until their body weight reached 30 g around P15 when they received the adult dose of 50 mg/kg.

Seven days after stroke induction, rats received iPSC-NPC transplantation. Under 4% chloral hydrate anesthesia, the skull over the penumbra was thinned by a dental drill. The stroke tissue was visually identified through the skull. 400,000 iPSC-NPCs were delivered through 4 x 1 μl injections at four sites in the peri-infarct region (+/-1 mm of bregma) using a microinjector (Nanoject II, Drummond Scientific, Broomall, PA). Cells were labeled with Hoechst 33342 (1:10,000) in the media for 1h prior to transplantation. Stroke only control animals received 4 x 1 μl injections of SATO media at four sites as a vehicle control. The incision was closed up with surgery adhesive. At 14d days after transplantation, the animals were anesthetized and decapitated. The brain was dissected from the cranium, a 5-mm coronal section including the injury was flash frozen on dry ice in OCT mounting media.

Mice:

To induce focal cerebral ischemia in the adult mice, the mice were anesthetized with 4% chloral hydrate. Surgery commenced when the mouse showed no response to pinches to test reflexes. The bilateral common carotid arteries (CCA) were temporarily occluded for 7 minutes with suture tie and the distal branch of right middle cerebral artery (MCA) was permanently occluded to produce an ischemia in the right whisker barrel cortex. Sham animals received skin incisions, but no arterial occlusions. Mice would receive iPSC-NPC transplantation 7d after stroke. iPSC-NPCs were labeled with Hoechst 33342 (1:10,000) in the media for 1h prior to transplantation. iPSC-NPCs were harvested and transplanted as either 100,000 cells/μl or 300,000 cells/μl in 4μl divided 2 injections in to the peri-infarct region. The animals were anesthetized with 4% chloral hydrate until they were in a deep anesthetic state. The incision over the right side of stroke was opened and the skull was thinned over the area to be injected. 2 injections of 2ul of cells were delivered slowly into the different sites around the stroke which was identified visually. Stroke only control animals received 2 x 2 μl injections of SATO media at 2 sites as a vehicle treatment. For one group of experiments, mice received 2 SDF-1 protein injections into the peri-infarct cortex (600ng/mouse) On the day of transplantation until sacrifice, mice received a daily intraperitoneal (i.p.) injection of Bromodeoxyuridine (BrdU; 50 mg/kg Sigma) to label proliferating cells.

Western Blot on iPSC-NPCs and Peri-infarct Brain Tissue

To examine trophic factors and receptors expressed by neural progenitors in culture, neural progenitors were collected after the differentiation. To analyze the trophic

factors in the peri-infarct tissue after cell transplantation, brain tissues were dissected from the peri-infarct area immediately after sacrifice and total protein was extracted using lysis buffer. Cells or tissues were homogenized with lysis buffer containing protease inhibitor (1:100). The bicinchoninic acid (BCA) assay was used to measure and standardize total protein concentrations for each sample. Protein samples for gel electrophoresis were created by combining the original protein solution with water and 5x loading buffer. Samples were boiled for 10 min at 95ºC. The samples were run on a 7.5-20% gradient acrylamide gel. For culture dishes, primary antibodies probed for VEGF (05-443; Millipore), VEGFR-2 (SC315; Santa Cruz), VEGFR-3 (AB1875; Millipore), BDNF (SC546; Santa Cruz), Angiopoietin-3 (Ang3, PC671; Oncogene Research Products, Cambridge, MA), and SDF-1α (MAB350; R&D Systems). For tissue samples, primary antibodies probed for VEGF (05-443, 1:1000; Millipore), SDF-1α (MAB350, 1:500; R&D Systems), β-actin (A5441 1:5000; Sigma), and tubulin (Cell signaling, Boston, MA). For cultures, blots were incubated in AP-conjugated secondary antibody anti-mouse, anti-rabbit, or anti-goat (1:1000; Promega, Madison, WI). Tissue SDF-1α blots were incubated in AP-conjugated secondary antibody anti-mouse (1:1000; Promega) at room temperature, washed and developed with NBT/BCIP solution then washed with water to stop the reaction. The tissue VEGF blot was incubated in horseradish peroxidase (HRP)-conjugated secondary antibody anti-mouse (1:1000; Bio-Rad) to be visualized by film. The HRP fluorescence was developed with Pierce enhanced chemiluminescence (ECL) Western Blotting substrate (Thermo Scientific, Waltham, MA) and immediately exposed to film and developed. All blots and film were

quantified with Image J (NIH). Values for each band were normalized against the β-actin or tubulin loading control.

Immunohistochemistry

In our in vivo analyses, fresh frozen brains were sectioned at 20μm for rat tissue and 10μm for mouse tissue on a cryostat microtome at -20°C using design-based stereology in which every tenth section was collected such that two adjacent tissues were more than 200μm apart. This method avoided double-counting a cell in two adjacent sections. Sectioned tissues were fixed with 10% buffered formalin and treated a -20°C ethanol/acetic acid solution. They were permeabilized with a 0.2% Triton-X 100 solution, and blocked for non-specific binding with 1% fish gel (Sigma). Tissues and cells were washed three times with PBS between each step. Tissues were incubated overnight at 4°C with primary antibodies for BrdU (OBT0030G, 1:400; AbD Serotec, Hercules, CA), NeuN (MAB377 1:200; Millipore), Collagen IV (AB769, 1:200; Millipore) or Glucose transporter 1 (Glut-1), and Glial fibrillary acidic protein (GFAP) (MAB360, 1:200; Millipore). After washing with PBS, secondary antibodies conjugated to fluorophores were used to visualize BrdU (donkey anti-rat Cy3, 1:300; Jackson ImmunoResearch), NeuN (donkey anti-mouse Cy5, 1:200), Collagen IV (donkey anti-goat AlexaFluor 488, 1:200; Invitrogen Life Technologies), and GFAP (donkey anti-mouse Cy3, 1:200; Jackson ImmunoResearch). The secondary antibodies were applied and incubated on the tissue for 1 hour at room temperature and subsequently washed 3 times with PBS.

Stained tissues and cells were analyzed under fluorescent microscopy. Pictures from six areas in the peri-infarct area (based on the border between live and dead NeuN-

positive cells adjacent to the ischemic core) were taken at 40x. Six tissue sections for each animal were quantified. NeuN/BrdU co-labeled and Collagen IV/BrdU or Glut-1/BrdU co-labeled cells were counted per animal.

Hematoxylin & Eosin (H&E) Staining

To visualize tissue growth indicative of tumorigenesis after iPSC-NPC transplantation, we stained the tissues with H&E. Tissues were dissected, processed, and sectioned coronally in the same way as for immunohistochemistry described above. Tissues were heated on the slide warmer for 15 min and immersed in Gill's #1 Hematoxylin (Accustain, GHS-116-500ml; Sigma) for 4 min. Tissues were washed with tap water for 5 min and immersed to blue in 0.25% ammonia hydroxide for 1 min. Tissues were washed with tap water for 5 min then dipped in 1% Eosin Y for approximately 5 sec. A series of increasing ethanol rinses were used to dehydrate the tissues. Tissues were immersed for 5 min each in 85%, 95%, and 2 times in 100% ethanol. Tissues were immersed in xylene for 5 min and cover-slipped with VectaMount permanent mounting medium (Vector Laboratories).

Vibrissae-Elicited Forelimb Placement Behavior Test

The vibrissae-elicited forelimb placement test is a test to directly assay whisker somatosensation and forepaw motor function[212,213]. Without impeding the forelimb movement, the rat was scruffed loosely to brush either right or left whiskers on a table edge. The animal was held in a horizontal position and passed from above the table edge to below the table edge once. Upon stimulation of the whiskers, the normal rat with no

brain injury will stereotypically reach out to the ledge. Rats with no deficit will reach out approximately an equal amount of times for both sides of whisker stimulation. A rat with a stroke deficit will reach out fewer times when the whiskers contralateral to the injury have been stimulated. Animals were trained 2 times on each side before testing. Each animal was tested for 6-10 times on each side and the percentage of successful reaches was calculated.

Top Scan Behavioral Tracking

The Top Scan software from CleverSys, Inc. was used to track animal migration distance, time spent staying still (characterized when <5% of body pixels showed movement), and migration velocity in a testing chamber (50cm x 50cm). 7d and 14d after stroke, mice with control or SDF-1 cell transplantation were placed into the testing chamber and recorded by the tracking system for 1h. The data was analyzed and graphed via Prism Graphpad software.

Adhesive Removal Test

The adhehsive removal test was performed following previous protocol[214]. An adhesive sticker is placed on the right or left paw at random for 4 trials. Time-to-notice the sticker and time-to-remove are recorded. The animal will take more time to notice and remove the sticker from the deficient contralateral paw compared to the ipsilateral paw. The 4 trials for the left and right paw notice and removal will be averaged and compared with others in the same treatment group. Data was analyzed with completely randomized analysis of variance (ANOVA) design. We expect that sham groups will

exhibit the least deficient behavioral results (i.e. more left turns and less time to notice and remove sticker). We expect that the iPS cells expressing SDF-1 will have the next highest behavioral results. Mice were trained and acclimated to the adhesive removal test equally to all treatment groups at least one day before testing.

Chapter VI

iPS Cell Transplantation Increases Regeneration and Functional Activities in a Model of Neonatal Ischemic Stroke

A. Introduction

Ischemic neonatal/perinatal stroke is a devastating disease. In the U.S., perinatal stroke occurs at a rate of 1 out of 3500 live births each year[1,215]. The most common cause of arterial ischemic stroke in children ages 0-15 years is cerebral arteriopathy which comprises more than half of all cases[1,216]. Among the causes of neonatal ischemic stroke are angiopathies and thromboembolism from an intracranial or extracranial vessel. Treatments available are very limited for ischemic stroke. Tissue plasminogen activator (tPA), a thrombolytic agent is the only FDA-approved drug used for treating ischemic stroke in adults[217]. The efficacy and safety of tPA in children is unknown. Currently, children with thrombosis are treated with anticoagulation using unfractionated heparin or low-molecular-weight heparin (LMWH) as standard therapy[218]. Stroke in the young can result in irreversible outcomes such as cognitive impairment, spasticity, or death[217]. Children who have suffered a perinatal stroke also are at risk for delayed effects such as seizures. Over 25% of survivors that experience a perinatal stroke develop a seizure within 3 years[219]. The medical cost of perinatal strokes is a financial strain to patients and society[220]. Thus, the need to alleviate the burden of pediatric ischemic stroke is great. This study focused on neonatal ischemic stroke using a rat model to test a regenerative strategy of cell transplantation therapy after neonatal stroke.

The postnatal brain has the ability for endogenous regeneration including neurogenesis and angiogenesis after ischemic stroke[77-80]. Neural progenitors are generated in the subventricular zone (SVZ). As a physiological process, the SVZ cells migrate to the olfactory bulb via the rostral migratory stream to differentiate into granule interneurons[81]. After ischemic stroke, chemoattractants such as SDF-1α in the injury and

peri-infarct region are highly upregulated and divert neural progenitors from the rostral migratory stream towards the ischemic infarct[66,122,123]. Cerebral ischemia dramatically increases SVZ neural progenitor cell proliferation, migration to the infarct, and differentiation into neurons[78,125,127-130]. However, a large proportion of these progenitors and new neurons do not survive upon reaching the injury site due to the inflammatory and cytotoxic microenvironment surrounding the stroke[83]. Thus the endogenous regenerative activities and their abilities in tissue repair are limited and cannot fully repair the damaged brain tissues.

In the field of regenerative medicine, transplanting exogenous cells such as neural progenitors derived from iPSCs has provided promising potential for enhanced tissue repair and functional recovery after ischemic stroke[150]. iPSCs are pluripotent stem cells generated from adult somatic cells via the upregulation of four transcription factors, Oct3/4, Sox2, c-Myc, and Klf4[165]. The use of iPSCs circumvents the ethical and controversial issues surrounding the use of human embryonic stem cells. These cells have great potential for personalized medicine in which iPSCs can be generated using the patient's own somatic cells such as skin fibroblasts and avoid the immune rejection issues the body faces after transplantation.

In contrast to multipotent bone marrow mesenchymal stem cells (BMSCs), iPSCs can be differentiated into cells of any of the three primary germ layers[171] such as neurons and glia[221-223]. Although having great therapeutic potential, the profile of the major trophic factors expressed by iPSC-NPCs and their regenerative abilities after transplantation into a neonatal ischemic brain have not been delineated. The present investigation is the first

effort in focusing on the regenerative properties of neural progenitors derived from iPSCs in a neonatal ischemic stroke model in rats.

B. Results

The experimental timeline is delineated in Figure 6.1

Differentiation of Mouse iPSCs Into Functional Neurons

Mouse iPSCs in the maintenance stage stained positive for pluripotent markers, Sox2 and Oct3/4 (Figure 6.2 A-F). iPSCs were maintained on MEF feeder layers (Figure 6.2 G) and were differentiated in suspension culture into neural progenitors by a 4-/4+ RA differentiation protocol[169]. The pluripotent cells in suspension formed embryoid bodies one day after differentiation and remained in sphere aggregates throughout the differentiation process until harvest at day 8 (Figure 6.2 H). After harvesting the aggregates via trypisinization, the cells were plated on PDL/laminin-coated dishes. Counts of cells at 1d after harvest indicated 96.9% of cells expressing the neural progenitor marker, DCX (DCX-positive cells/total Hoechst cells) (Figure 6.2 I, J). Five days after harvest, cells exhibited neuronal morphology including processes projecting from the cell body (Figure 6.2 K, L).

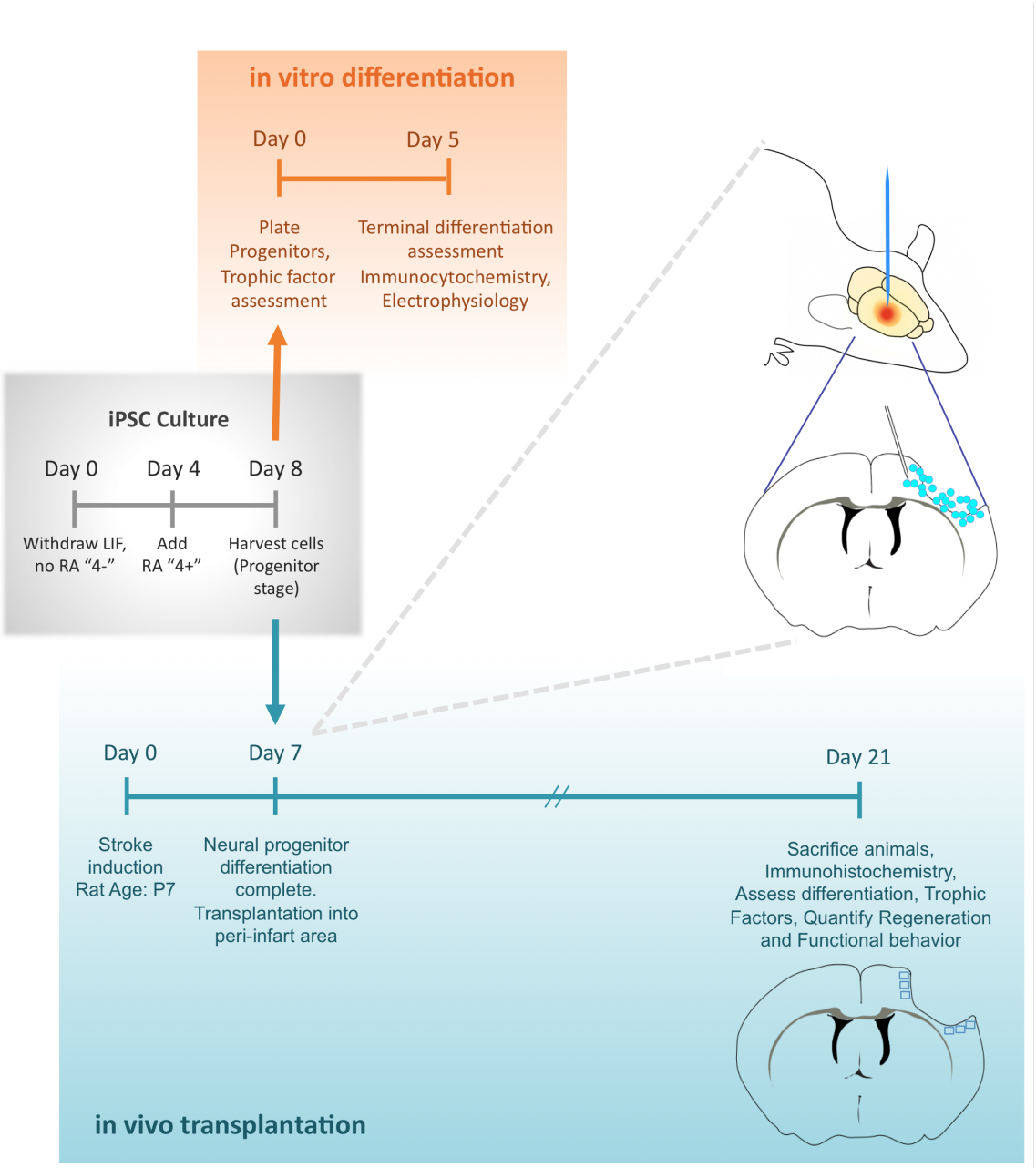


Figure 6.1. Experimental Timeline of In Vitro Differentiation and In Vivo Transplantation. This experimental timeline delineates the in vitro differentiation from the in vivo transplantation. iPS cells were differentiated in suspension culture with the retinoic acid protocol. Neural progenitors were harvested and were either plated and allowed to terminally differentiate or were transplanted into neonatal stroke rats

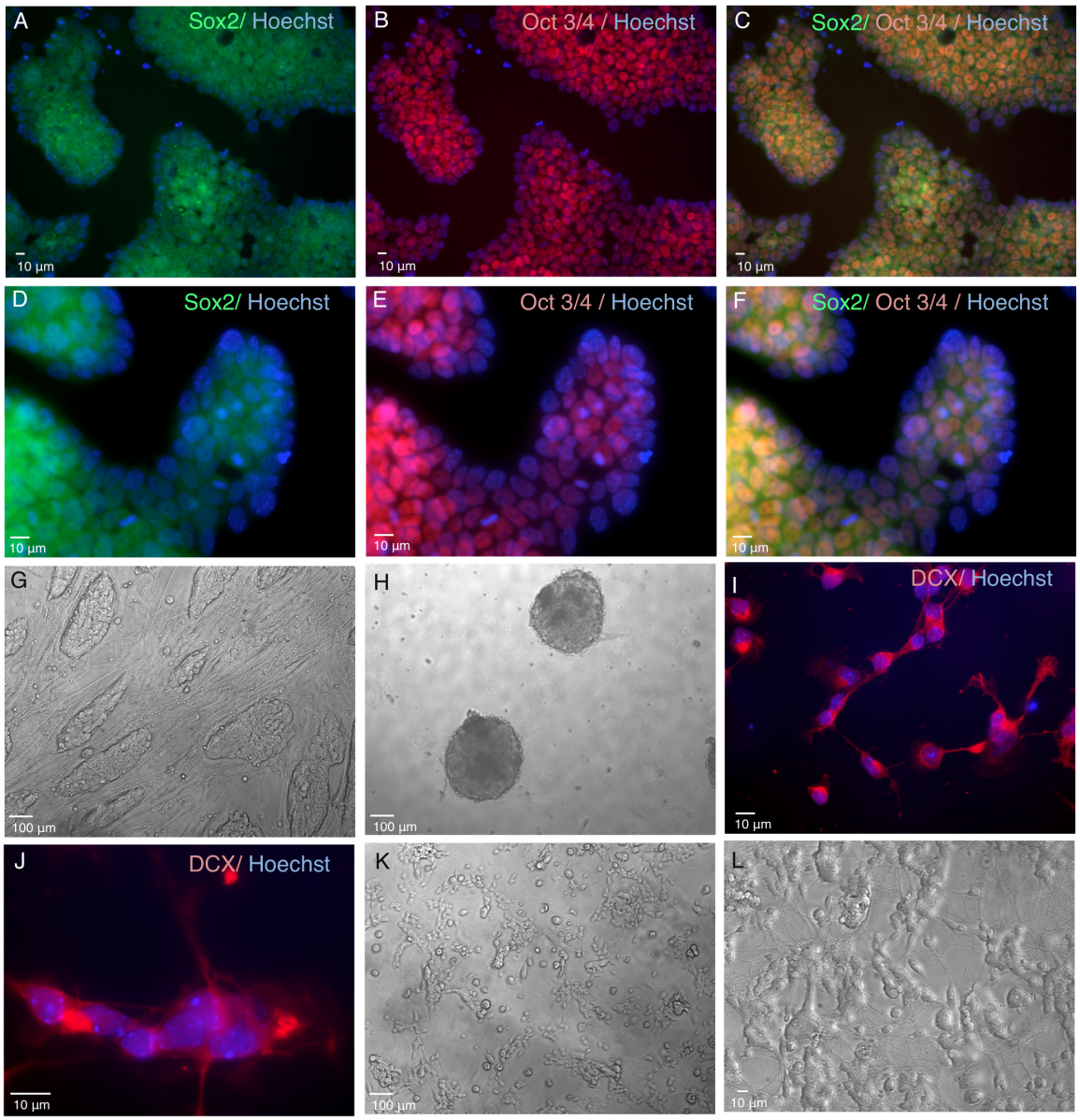


Figure 6.2. Pluripotent iPSCs Are Differentiated Down the Neural Lineage
A-F. iPSCs stained positive for Sox2 and Oct3/4 **G.** iPSCs were maintained on a feeder layer **H.** The pluripotent cells in suspension formed embryoid bodies during differentiation. **I-J.** 1d after harvest, the cells stained positive for DCX **K-L.** 5d after harvest, cells exhibited neuronal morphology including processes projecting from the cell body

To verify the formation of mature neurons and neuronal activity, we stained cells for mature neuronal markers, Tuj-1, NeuN, and neurofilament and performed electrophysiological recordings on these cells (Figure 6.3 A-F, H, I). At 5d after harvest, 87.5% of cells expressed NeuN (NeuN-positive cells/total Hoechst-labeled cells) (Figure 6.3 C, H-I). Also at 5d, cells expressed SNAP-25, synapsin 1 and neurofilament-positive staining (Figure 6.3 D-I). Whole-cell patch clamp recordings were performed 10d after harvest. Differentiated cells exhibited neuronal functionality with action potentials triggered by a 30μA current injection (average amplitude = 53.31± 12.77 mV, n= 7 cells). Inward Na^+ current and outward K+ current were elicited by step voltage changes from a holding potential of -70 mV to depolarized levels (-40 mV to +50mV in 10 mV decrement). The inward Na+ current was highly sensitive to block by the specific channel blocker tetrodotoxin (TTX, 1μM; Figure 6.3 A).

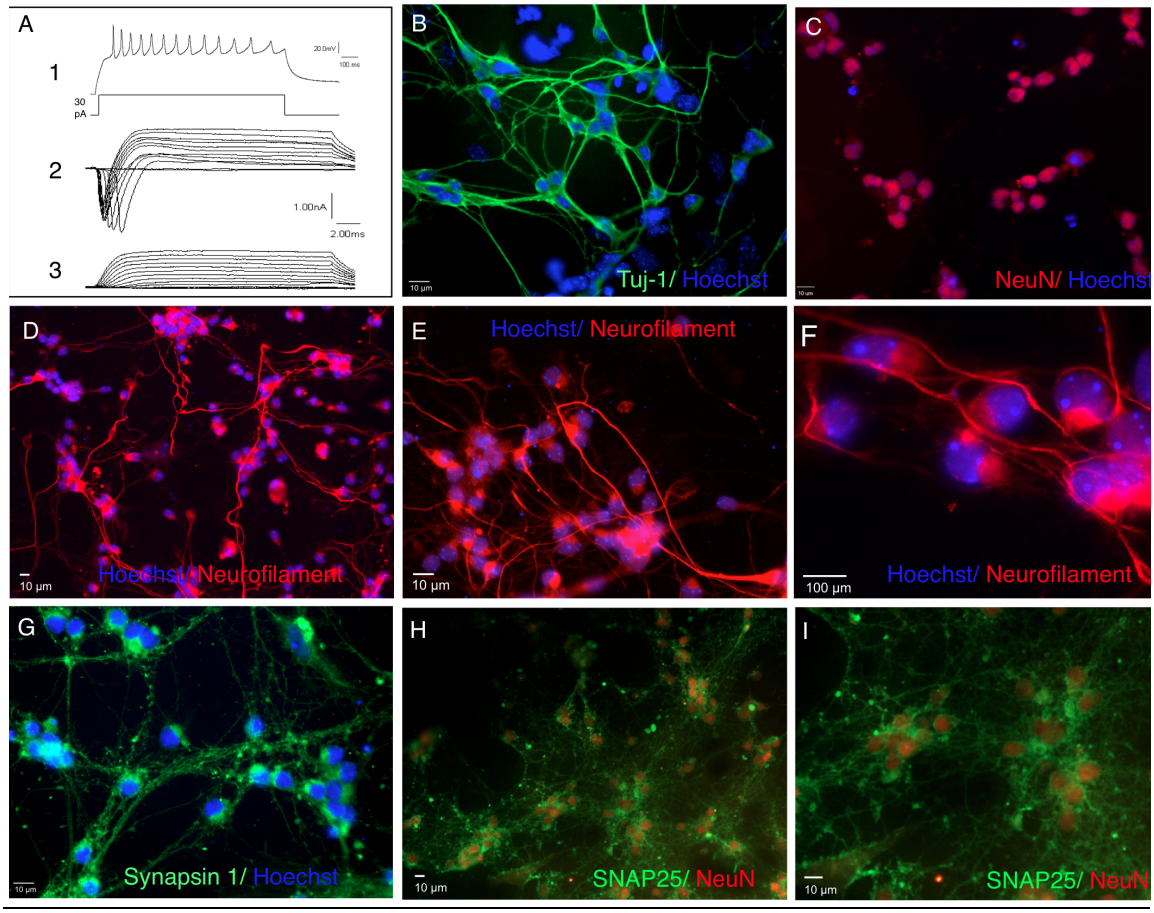


Figure 6.3. iPSCs Are Differentiated into Functional Mature Neurons
A. iPSC-derived neurons exhibited neuronal functionality with action potentials, and functional Na^+ and K+ channels. When TTX blocker was applied, I_{Na} was completely blocked. **B-F.** To verify neuronal differentiation, cells were stained for mature neuronal markers, Tuj-1, NeuN (87.5% differentiation rate), and neurofilament **G-I.** Cells also expressed SNAP-25 and synapsin 1

3. iPSC-NPCs Express Trophic Factors In Vitro

Using immunocytochemistry, we detected the expression of BDNF, FGF, SDF-1α, GDNF, and EPO in iPSC-NPCs (Figure 6.4 A-G). We verified this trophic factor expression and the expression of VEGF and CXCR4 with PCR analysis of neural progenitors (Figure 6.4 H). In addition, the protein expression of several angiogenic

trophic factors including VEGF, BDNF, SDF-1α, Ang3 and the receptors for VEGF including VEGFR-2 and -3 were detected using Western blot analysis (Figure 6.4 I).

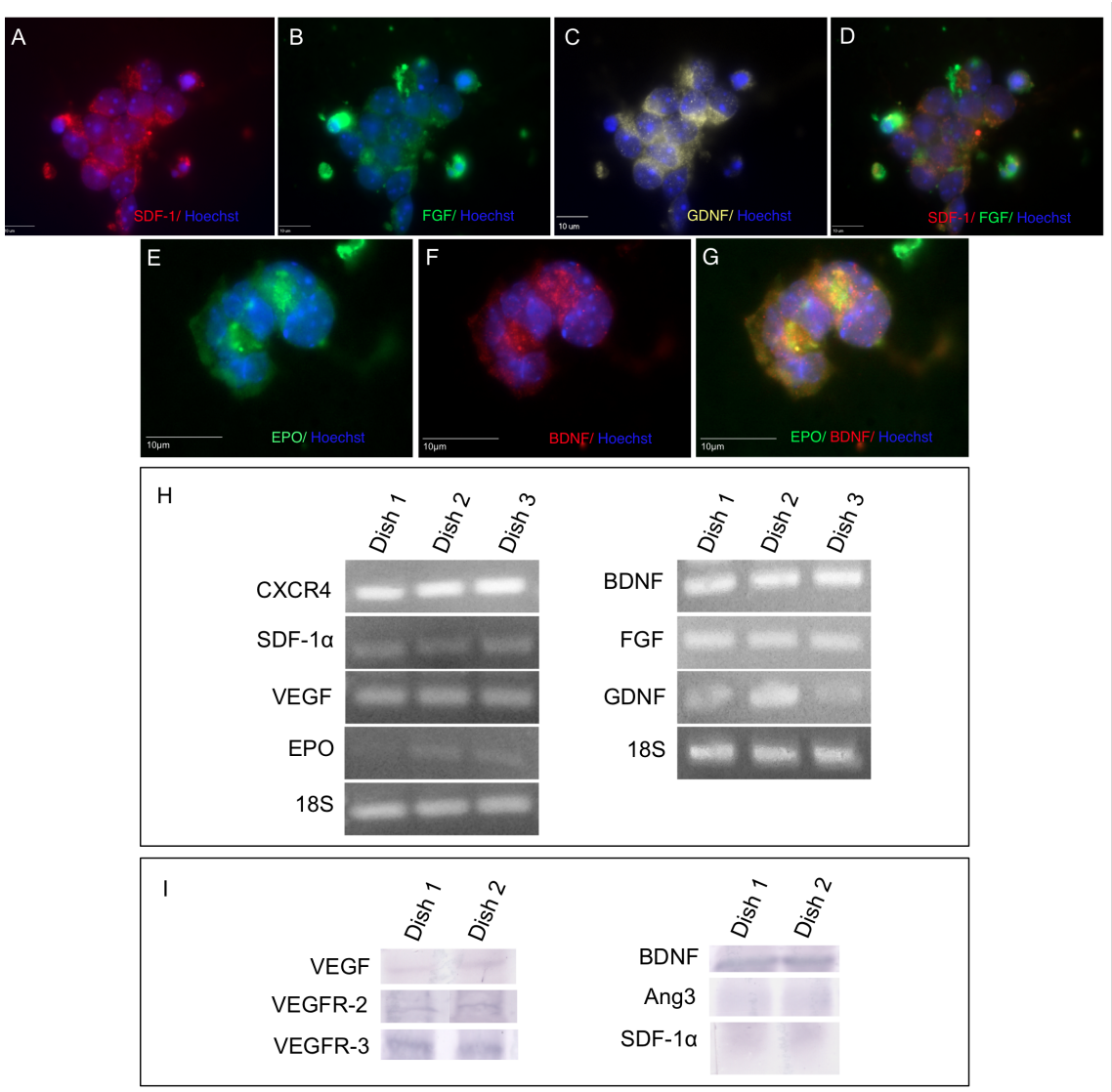


Figure 6.4. Differentiated iPSCs Express Trophic Factors
A-G. iPSC-NPCs expressed BDNF, FGF, SDF-1α, GDNF, and EPO through immunohistochemistry. **H.** Trophic factor expression of BDNF, FGF, SDF-1α, VEGF, GDNF, EPO, and CXCR4 with PCR analysis of iPSC-NPCs **I.** Several angiogenic trophic factors were detected through Western blot including VEGF, BDNF, SDF-1α, Ang3 and the receptors for VEGF including VEGFR-2 and -3.

Transplantation of iPSC-derived Neural Progenitor Cells After Stroke Increases Trophic Factors

We tested if transplanting iPSC-NPCs could increase the levels of two major trophic factors, SDF-1α and VEGF in the peri-infarct tissue after ischemic stroke. Tissue from the peri-infarct area was collected 2 days after iPSC-NPCs were transplanted. Western blot analysis demonstrated that, as previously reported, SDF-1α expression in the peri-infarct area increased after ischemic stroke[66]. Animals with cell transplantation showed a significant increase in SDF-1α expression compared to the sham animal group (Figure 6.5 A, n=3-4, p=0.004. Similarly, VEGF expression in the iPSC-NPC transplantation group was significantly greater than stroke and sham groups (p=3-4, p=0.0028).

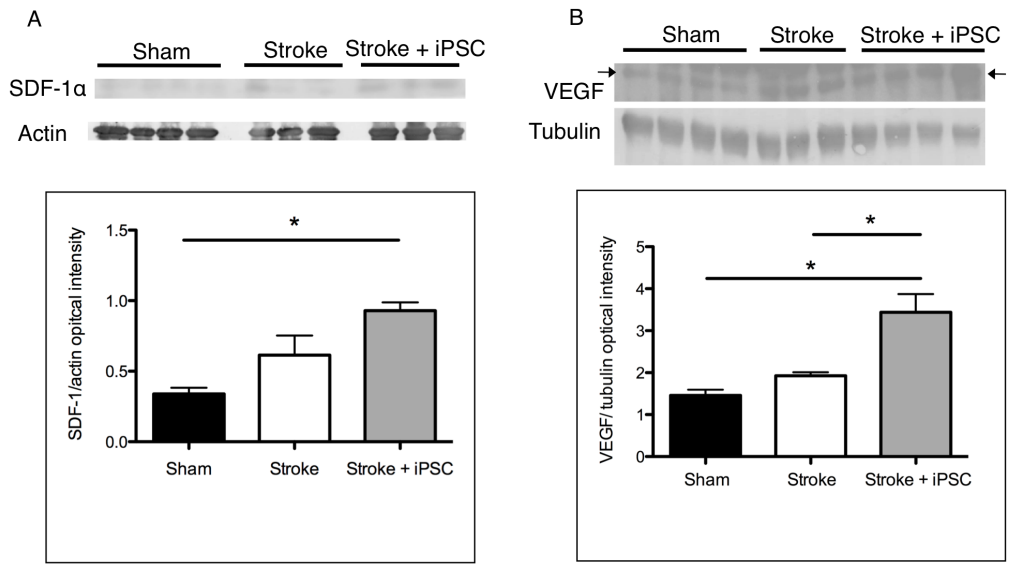


Figure 6.5. Increased Trophic Factor Expression After Transplantation
A. Tissues were dissected from the peri-infarct area 2d after iPSC-NPC transplantation. Animals with transplantation showed a significant increase in SDF-1α expression compared to the sham animal group (n=4, p=0.004). **B.** VEGF expression in the iPSC-NPC transplantation group was significantly greater than stroke only and sham groups (p=3-4, p=0.0028).

Transplanted iPSC-NPCs Differentiate Into Neural Lineage Cells

Stroke animals received iPSC-NPC transplantation (400,000 cells/animal) or media control 7 days after stroke. We chose a delayed 7-day time point to avoid the cytotoxic milieu and brain edema found in acute and sub-acute phases of ischemia similar to previous studies[159,224]. Much of the inflammatory response and edema subsided 7 days after stroke, which allows for a more hospitable environment for exogenous cell transplantation. We were able to see that transplanted cells differentiated into neural lineage cells at 14 days after transplantation. A portion of pre-labeled Hoechst 33342 transplanted cells had differentiated into GFAP-positive cells as indicated by the GFAP staining co-labeled with Hoechst (Figure 6.6 A-F). A portion of cells had also differentiated into NeuN-positive cells, indicated by the NeuN and Hoechst 33342 co-labeling (Figure 6.6 G-K). A group of 5 transplanted animals were saved for 4 months after transplantation to see whether or not tumors would form due to the transplantation. Using the H&E stain, no abnormal cell growth or tumors formation was detected at 4 months after transplantation (Figure 6.6 L).

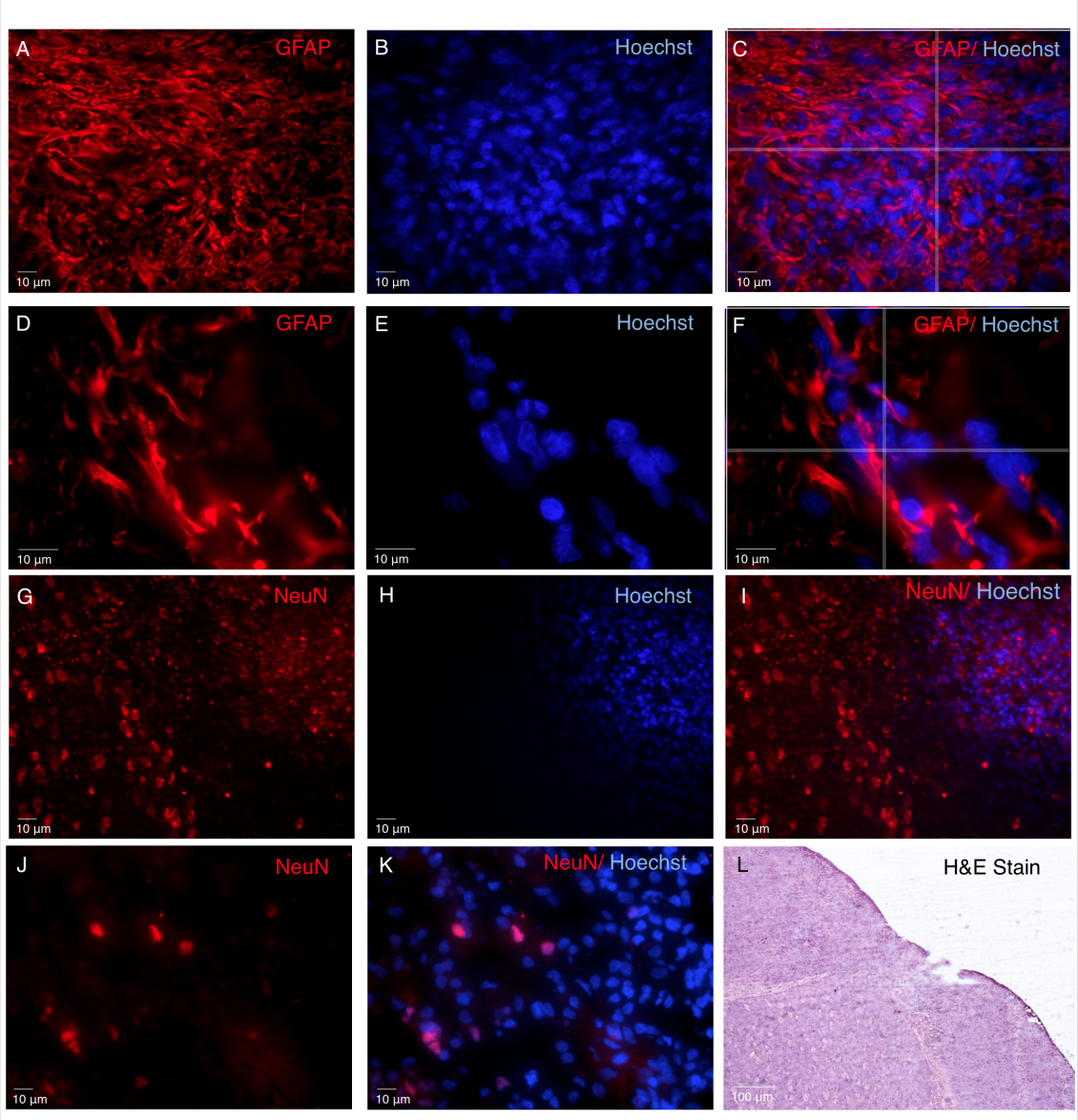


Figure 6.6. Transplanted iPSCs Differentiate into Neuronal-like Cells
A-F. A portion of cells had differentiated into GFAP-positive cells **G-K.** A portion of cells had also differentiated into NeuN-positive cells. **L.** A group of 5 transplanted animals were saved for 4 months after transplantation to test for tumorigenesis. No tumors formed at 4 months after transplantation

Transplantation of iPSC-NPCs Increased Endogenous Neurogenesis and Angiogenesis

Dissected brain tissues were analyzed for neurogenesis and angiogenesis 21 days after stroke when active endogenous neural progenitor proliferation and migration activity are observed post-ischemia[126]. Animals were injected with BrdU (i.p.) starting 3d after stroke to label newly formed neuronal cells (NeuN-positive) and proliferating vessels (Collagen IV-positive cells labeling the vessel basement membrane). In stroke only controls (Figure 6.7 A-D) and iPSC-NPC transplanted brains (Figure 6.7 E-H), we compared the number of co-labeled NeuN/BrdU cells (Figure 6.7 I-K) and co-labeled Collagen IV/BrdU cells in the peri-infarct area (Figure 6.7 L-N). There were significantly more NeuN/BrdU (n=7-9; p=0.0142) and Collagen IV/BrdU (n=7-9; p= 0.0404) co-labeled cells in iPSC-NPC transplantation group than in the stroke only group (Figure 6.7 K, N).

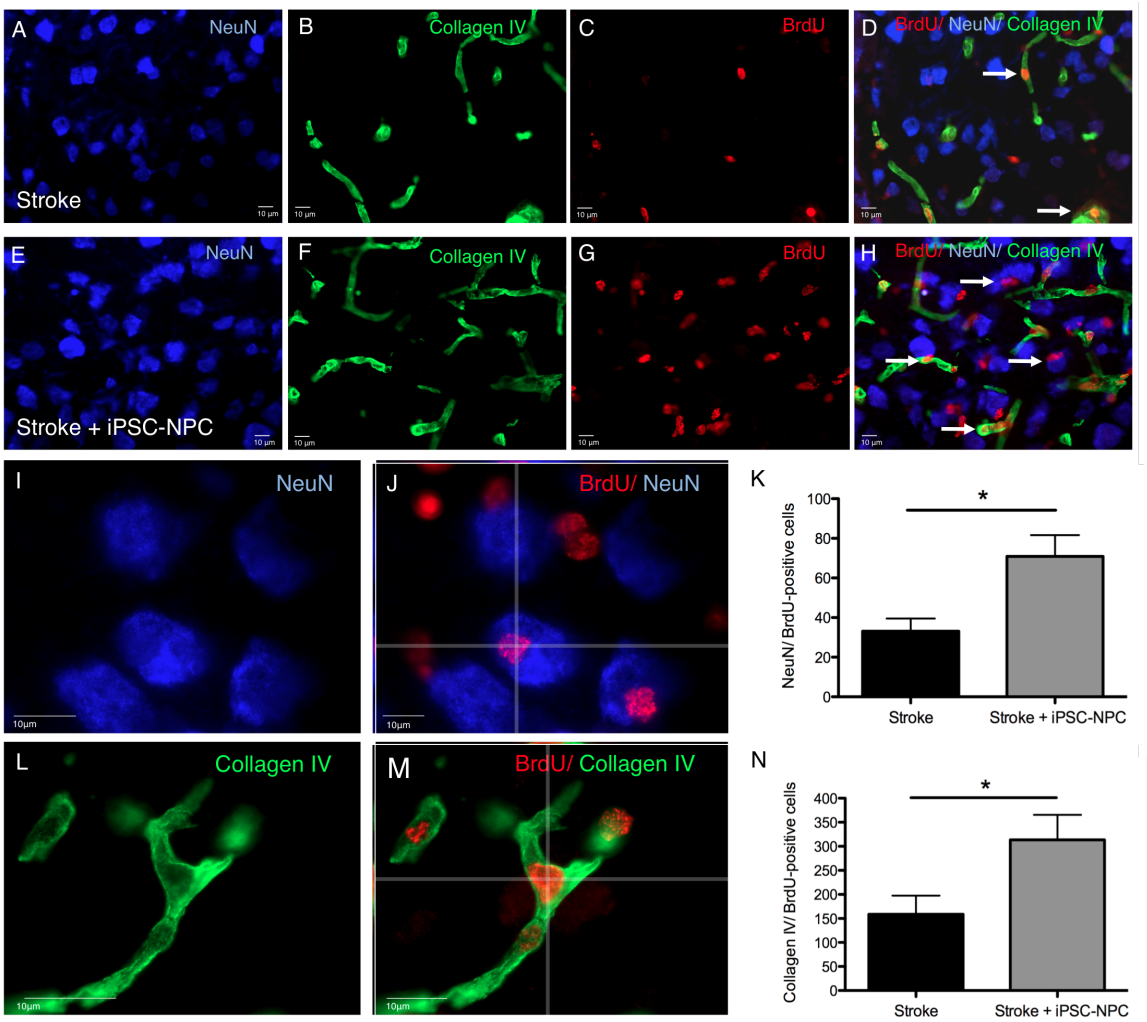


Figure 6.7. Transplantation Increases Regeneration
Animals were injected with BrdU i.p. 3d-21d after stroke in animals with: **A-D.** stroke and **E-H.** stroke+ iPSC-NPC transplantation. White arrows indicate co-labeled cells. **I-K.** There was a significant increase in number of co-labeled NeuN/BrdU cells after transplantation compared to stroke control (n=7-9; p=0.0142). and **L-N.** co-labeled Collagen IV/ BrdU cells in the peri-infarct area after transplantation compared to stroke control (n=7-9; p= 0.0404)

Transplantation of iPSCs In Vivo Increases Functional Recovery

Our stroke model produces a consistent injury in the sensorimotor cortex that includes the whisker barrel cortex in the neonatal rats[64]. We analyzed the somatosensory function of sham, stroke, and stroke plus iPSC-NPC transplantation animals using the vibrissae-elicited forelimb placement test 14 days after treatment (21 days after the onset

of stroke). No tests were performed before the stroke induction because the P7 neonatal animals had not developed the proper motor abilities to perform this test. Sham and stroke vehicle animals at the same time point served as controls. In the vibrissae-elicited forelimb placement test, the number of successful reaches of the right (corresponding to the contralateral cortex) and left (corresponding to the ipsilateral cortex) paws were quantified as a percentage of total trials performed (Figure 6.8). Sham animals had a similar (not significantly different) percentage of successful reaches with their left and right paws. Animals with stroke exhibited a significantly lowered percentage of successful reaches on the left/impaired side compared with shams (Figure 6.8, n=7-9; p= 0.0315). Animals that had received iPSC-NPC transplantation showed no significant difference from sham animals (Figure 6.8).

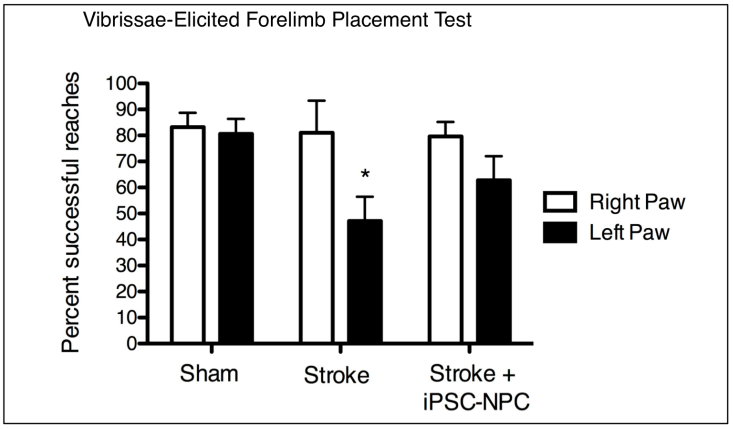


Figure 6. 8. Transplantation Increases Functional Recovery
In the vibrissae-elicited forelimb placement test, sham animals have a similar percentage of successful reaches between the left and right paws. Animals with stroke exhibited a significantly lowered percentage of successful reaches on the left side compared with shams (n=7-9; p= 0.0315). Animals with iPSC-NPC transplantation exhibit an increase in left side compared to stroke animals not significant to sham animals.

C. Discussion

iPSC transplantation into a stroke model has shown beneficial regenerative and recovery effects in previous studies[150,159,172,224], but its trophic factor expression and regenerative capabilities have not been fully evaluated and especially little information is available in neonatal stroke. In the present investigation, we show in vitro and in vivo evidence that iPSC-NPCs can different into functional mature neurons. Differentiated cells express elevated levels of pro-regenerative factors that can stimulate regeneration observed in the peri-infarct region after neonatal ischemic stroke. In vitro studies were performed in parallel with in vivo transplantation in order to monitor the quality and differentiation of the batch of cells that were transplanted. We characterized the stages of neuronal differentiation of mouse iPSCs, profiled major trophic factors expressed by iPSC-NPCs, evaluated trophic factor levels after transplantation, demonstrated the ability of transplanted iPSC-NPCs to differentiation in vivo, and demonstrated increased angiogenesis and neurogenesis and functional recovery in rats received iPSC-NPC transplantation.

Using the RA differentiation protocol, we demonstrate the neural differentiation of Oct3/4 and Sox2-positive pluripotent iPSCs into DCX-positive neural progenitors with a 96.9% differentiation rate. Further, harvested cells terminally differentiated into neurons that expressed several neuronal markers including Tuj-1, neurofilament, and NeuN. The SNAP-25 and synapsin 1 expression of cells suggest that the cells have the machinery and thus the potential to build synapses and regulate synaptic activity. We confirm that mouse iPSCs can be differentiated into neural progenitors and neurons exhibiting functional electrophysiological activity. At 10d after harvest, cells exhibited

action potentials, and inward Na^+ and outward K^+ currents. Since it is important for cells to differentiate into functional neurons in order to replace lost neurons in the injury area, we hypothesized that transplanting iPSC-NPCs can provide more cells to the injury and also be reparative through their trophic factors.

Trophic factors found in stem cells have pleiotropic effects in that their transplantation after stroke enhances endogenous neurogenesis and angiogenesis. Studies have shown that many stem cell types express regenerative trophic factors. For example, mesenchymal stem cells (MSCs) have been shown to release a wide range of adaptive factors such as factors that are cytoprotective (endothelin), angiogenic (VEGF), and play a role in cell migration (LRP-1, LRP-6)[188]. To illustrate their paracrine effects, MSC-conditioned media increased neurite outgrowth and branch number when applied to neurons[187]. Similarly, iPSC have recently been shown to express trophic factors including VEGF in neuroepithelial-like stem cells derived from human iPSCs[172]. We profiled the regenerative trophic factors expressed by iPSC-NPCs. Using immunohistochemistry, we found that the iPSC-NPCs express SDF-1α, FGF, GDNF, EPO, and BDNF. PCR assays confirmed that iPSC-NPCs express these trophic factors as well as VEGF and CXCR4, a receptor of SDF-1α. These are all major angiogenic and neurotrophic factors that contribute to regeneration after stroke[94,132,194,225-227]. Western blot analysis of the iPSC-NPCs further confirmed expression of several angiogenic factors and their receptors including VEGF, SDF-1α, BDNF, Ang3, VEGFR-2, VEGFR-3. The SDF-1α and VEGF receptors expressed by iPSC-NPCs may help to localize the transplanted cells to the injury area after transplantation to encourage local regeneration. To translate this aspect to in vivo, we transplanted these iPSC-NPCs into the peri-infarct area and demonstrated

that SDF-1α and VEGF levels increased in the peri-infarct tissue after transplantation. Previous studies in vitro have shown that VEGF stimulates the tubule formation of endothelial cells (human umbilical vein endothelial cells, HUVEC) resulting in an increase in vessels[190]. With the addition of VEGF antibody, this effect was attenuated[190]. In an in vivo example, transplanted neural stem cells that secreted VEGF showed an increase in neovascularization in the peri-infarct area demonstrating a greater blood vessel density in animals with transplantation compared controls at two weeks after transplantation[191].

Among its pleiotropic effects, trophic factors enhance neurogenesis and neural progenitor migration. Chemoattractive trophic factors like SDF-1α play an important role in homing endogenous neural progenitors to the stroke lesion[82,132]. SDF-1α is greatly upregulated in the infarct after stroke and can effectively chemoattract endogenous neural progenitors through its receptor CXCR4[66,116]. By blocking the SDF-1α signaling with a neutralizing antibody against CXCR4, migration was attenuated[132]. In the present investigation, iPSC-NPCs were transplanted during the delayed phase of stroke and supplemented the rising levels of SDF-1α in the infarct and the repair process after stroke[66,132]. SDF-1α is pleiotropic in that it also contributes to angiogenesis after stroke by attracting of endothelial cells for revascularization[192,193]. SDF-1α and VEGF both play roles in endogenous neural progenitor migration as well as endothelial progenitor cell recruitment[132,194]. Endogenous neural and endothelial cell progenitors express CXCR4 and are chemoattracted to SDF-1α and VEGF[116,195].

In the present study, we demonstrate that iPSC-NPCs can differentiate into neural lineage cells including glia and neurons suggesting an addition of replacement cells to the

peri-infarct area. We also addressed the tumorigenic potential of iPSC transplantation in this study. To evaluate whether or not transplantation led to tumorigenesis, we examined the brain tissue 4 months after transplanting iPSC-NPCs into the rats. This agrees with the current view that transplantation of undifferentiated iPSCs may lead to tumorigenesis[228], and that transplantation of neuronal lineage-induced ES and iPS cells is an effective way of avoiding tumor growth after transplantation.

We tested the endogenous regenerative potential after iPSC-NPC transplantation and observed a significant increase in NeuN/BrdU and Collagen IV/BrdU co-labeled cells at the peri-infarct area of animals with iPSC-NPC transplantation demonstrating an increase in neurogenesis and angiogenesis. An increase in the migration of neural progenitors and the increase in vessel cells could be the effect of trophic factors from the transplanted iPCS-NPCs. NeuN/BrdU co-labeling indicates that a neural progenitor that had differentiated into a neuron. The NeuN/BrdU cells originated as endogenous neural progenitors that had migrated and differentiated into NeuN-positive cells before or after arriving at the peri-infarct area. The source of the NeuN/BrdU cells are likely endogenous and not exogenous since iPSC-NPCs show low levels of proliferation after engraftment into the brain tissue and in culture after harvest[224].

The endogenous angiogenesis and neurogenesis observed in this study may be a remodeling of the damaged neurovascular unit. The neurovascular unit is a functional entity in the brain parenchyma where the CNS, comprising of neuronal cells, astrocytes, and pericytes interfaces with the vasculature of the circulatory system to maintain metabolic homeostasis such as ionic concentrations. This functional unit is severely compromised after an ischemic event, however with regeneration this unit may be

repaired. Previous work demonstrates that angiogenesis and neurogenesis are causally linked to regenerate the neurovascular unit[80]. For example, when angiogenesis was blocked with Endostatin after stroke, there was a correlative 10-fold drop of new neurons in the peri-infarct cortex[80]. Our observations in the present investigation of increased neurogenesis and angiogenesis after transplantation could be a synergistic event contributing to the rebuilding of the neurovascular unit.

To assess the functional recovery after iPSC-NPC transplantation, whisker somatosensation was tested with the vibrissae-elicited forelimb placement test. With iPSC-NPC transplantation, there was an increase in the percentage of reaches not significantly different than shams. Previous studies have shown that stem cell transplantation increases the functional recovery when transplanted into adult stroke[147,158] and neonatal stroke animals[151]. It has been suggested that transplanted cells may provide several benefits: a favorable trophic factor environment that attenuates cell death, increasing neurogenesis and angiogenesis after stroke, and the integration of transplanted cells into the existing circuitry to improve functional recovery. Thus, both exogenously transplanted iPSC-NPCs and endogenous NPCs contribute to the tissue repair after neonatal ischemic stroke. This study is the first to profile the many trophic factors expressed by iPSC-NPCs and how their transplantation increases angiogenesis and neurogenesis into a neonatal stroke model.

Chapter VII
The Role of SDF-1α Upregulation in Cell Survival and Differentiation In Vitro

A. Introduction

After ischemic stroke, SDF-1α is upregulated by cells in the infarct core as a response to hypoxic conditions and inflammation. SDF-1α is a 8-12 kda chemokine that plays a role in recruiting neural progenitors and endothelial progenitor cells to the infarct for tissue repair. This homing signal plays an important role in directing the migration of neural progenitors and vascular progenitors to the infarct for endogenous tissue regeneration.

SDF-1α is pleiotropic factor activating several divergent pathways to initiate cell migration, survival, proliferation, differentiation, and gene transcription. SDF-1α binds to the G-protein coupled receptor (GPCR), CXCR4 and activates multiple transduction pathways. The receptor is coupled to an intracellular heterotrimeric G-protein consisting of Gα, Gβ and Gγ subunits[140]. Upon the ligand binding to the receptor, the trimer dissociates intracellularly into a βγ dimer and the α monomer. There are different families of Gα subunit. Depending on the specific type of Gα subunit, the subunit can inhibit or stimulate adenyl cyclase which would increase or decrease cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA)[140]. The Gα, Gβ and Gγ subunits are the initiators to several pathways within the cell.

Some of the pathways of interest involve those activating chemotaxis of the cell. Chemotaxis activation in the cell involves the Gβ subunit which activates PLC to generate two second messengers, phospholipase C (PLC) and Inositol trisphosphate (IP3)[140]. Both of these increase the intracellular Ca^{2+} by mobilizing intracellular stores[229], thus SDF-1α binding to CXCR4 can result in greater intracellular Ca^{2+}. The increased Ca^{2+} can be measured and is correlated with chemotaxis[230]. Another pathway involved in

chemotaxis occurs with Gβγ and Gα subunits. Both Gβγ and Gα components can both activate PI3K. PI3K phosphorylates several focal adhesion factors including proline-rich kinase-2 (Pyk-2), Crk-associated substrate (p130Cas), focal adhesion kinase (FAK), and paxilin leading to chemotaxis[140,231,232]. These pathways are the mechanisms for the cellular migration observed upon SDF-1α binding in cell types that express CXCR4.

The activation of SDF-1α/CXCR4 can also activate pathways for cell survival. The aforementioned PI3K can lead to the activation of NF-KB for the transcription of pro-survival genes[140]. PI3K can also activate Akt which inactivates the apoptotic factor, Bcl-2-associated death promoter (BAD)[140]. Mitogen-activated protein kinases (MAPK) extracellular signal regulated kinases (MEK) can also inactivate BAD. Thus, cell survival upon SDF-1/CXCR4 activation can be mediated in two ways: genetically through the transcription of pro-survival factors or through the post-translational inhibition of cell death components.

Thus far, no work has examined the role of SDF-1α upregulation in iPSCs as a strategy to increase cell survival and trophic factor vehicle. In this chapter, we will demonstrate the feasibility of SDF-1α upregulation in iPSCs and the benefits of genetic upregulation of SDF-1α in iPSCs in vitro. We test the roles of SDF-1α in neuronal differentiation, migration, and cell survival. Characterizing these cells will provide a foundation for understanding the regenerative effects in the brain after cell transplantation. By upregulating SDF-1α in transplanted cells, we aim to stimulate the chemotaxis of endogenous progenitors as well as provide replacement cells to the injury.

B. Results

1. GFP-SDF-1α Can Be Expressed Under Different Promoters

GFP-SDF-1α was subcloned under several different promoters: CMV, CAG and, EF1α. Using a lipid-based transfection technique, we were able to successfully transfect all plasmid constructs into pluripotent iPSCs (Figure 7.1). GFP expression in the cell indicated successful uptake of the GFP-SDF-1α plasmid. Amongst these plasmids, we chose to use EF1α to create a stable cell line (Figure 7.1 D, H, L).

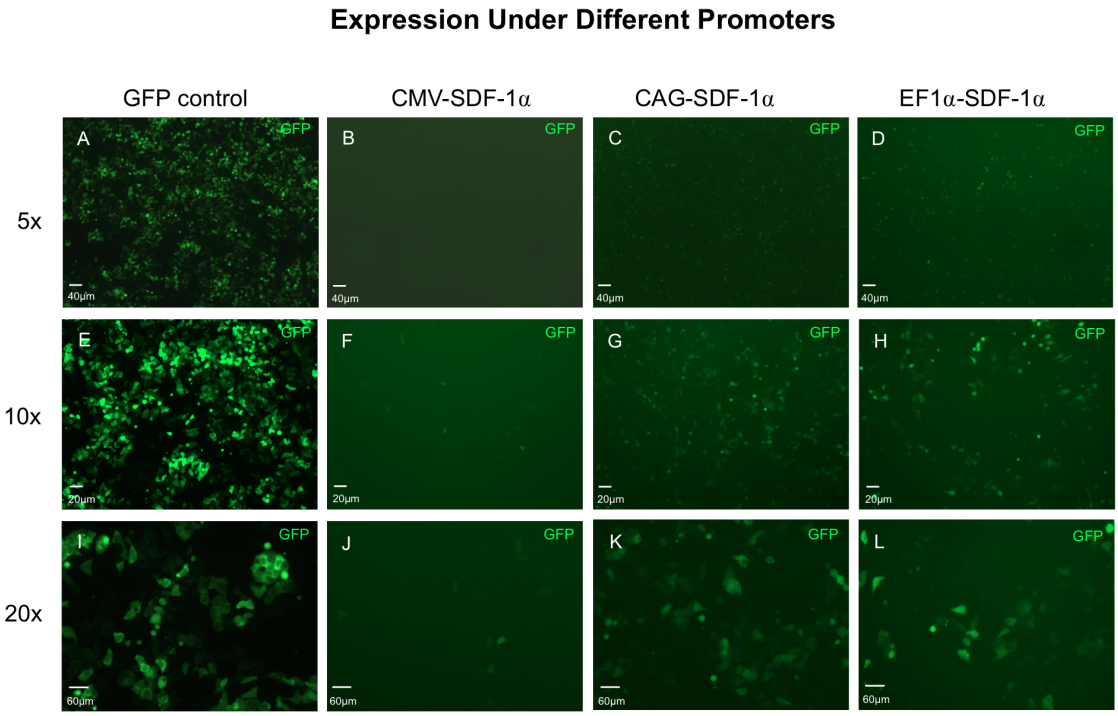


Figure 7.1. GFP-SDF-1α Can Be Expressed Under Different Promoters.
GFP-SDF-1α was subcloned under several different promoters, CMV, CAG, and EF1α. Using Lipofectamine 2000, 3 different plasmids were transfected into iPSCs to evaluate efficiency. 24h after transfection, all the promoters expressed GFP-SDF-1α. The EF1α plasmid had the best transfection efficiency and the CMV plasmid had the worst transfection efficiency. **A-D.** Pictures captured 24h after transfection at 5x magnification, **E-H.** 10x magnification, and **I-L.** 20x magnification

2. SDF-1α Can Be Stably Expressed in iPSCs

Stable cell lines were created with lentivirus containing the control plasmid and lentivirus containing the SDF-1α gene. The cells were selected via puromycin resistance selection marker to create a stable cell line. As a result, both the control (Figure 7.2 A-D) and SDF-1α cell (Figure 7.2 E-H) populations expressed GFP under fluorescent microscopy and retained GFP expression during neuronal differentiation in neurospheres (Figure 7.2 D, H). To test the upregulation of SDF-1α, reverse transcriptase-PCR was performed for SDF-1α and 18S control, and run out with electrophoresis (Figure 7.2 I). Cells with 0.5 μl, 5 μl, and 10μl application of SDF-1α lentivirus exhibited a greater SDF-1α expression compared to cells with 0.5 μl, 5 μl, and 10μl application of the control plasmid lentivirus (Figure 7.2 I). The SDF-1α expression of SDF-1α cells at each dosage was at least 5 times greater compared to control cells (Figure 7.2 J).

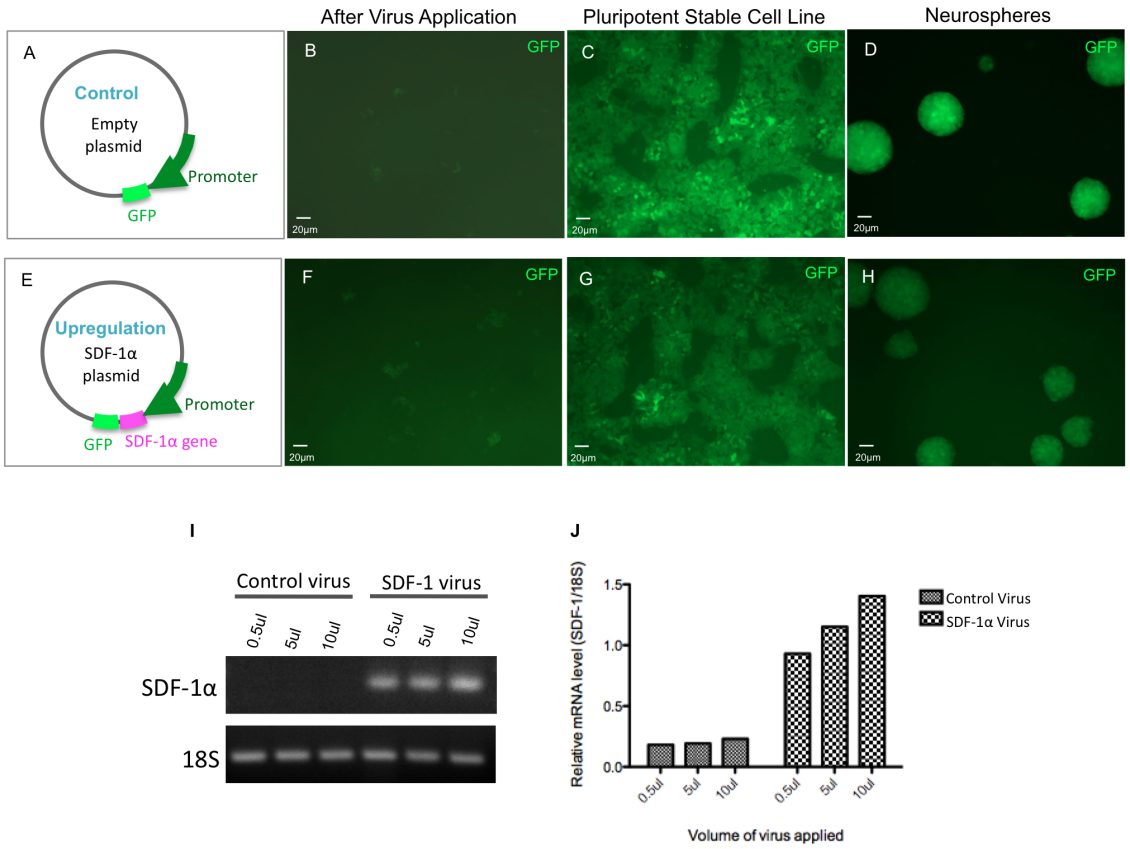
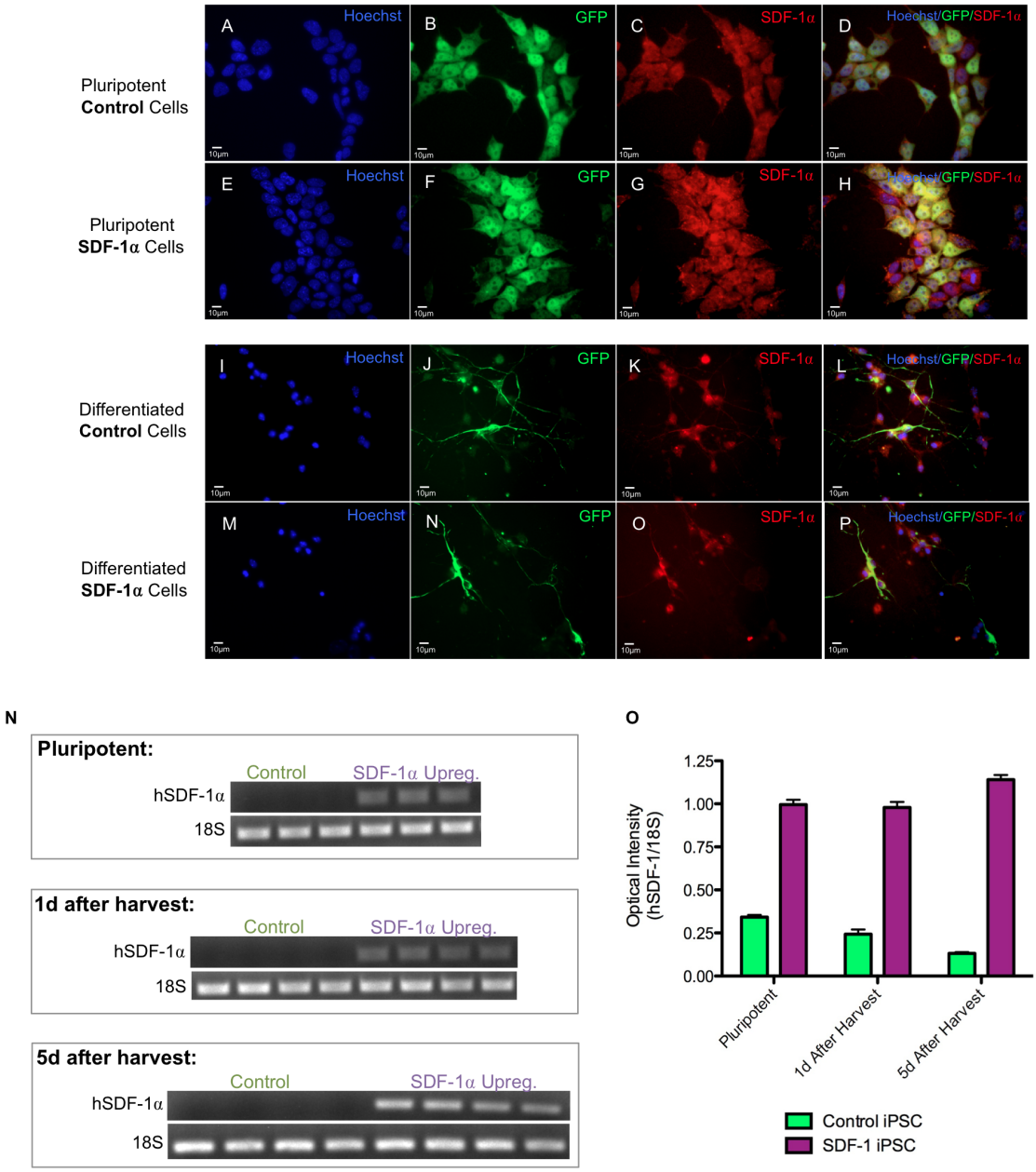


Figure 7.2. SDF-1α Can Be Stably Expressed in iPSCs
A-B, E-F. Pluripotent iPSCs were infected with a control or SDF-1α plasmid packaged into lentivirus and differentiated. **C-D, G-H.** Puromycin was used for selection to create a stable cell line to be differentiated in suspension culture as neurospheres. **I-J.** PCR confirmed that SDF-1α upregulation was present and dose-responsive.

3. SDF-1α Expression is Maintained Throughout the Stages of Neuronal Differentiation

SDF-1α and control pluripotent iPS cells both express GFP after virus transduction. Both pluripotent cells lines express SDF-1α and GFP (Figure 7.3 A-D, E-F). After differentiation, both SDF-1α and control lines also retain SDF-1α and GFP expression (Figure 7.3 I-L, M-P). PCR confirms that the upregulation of SDF-1α is retained throughout the pluripotent, progenitor (1d after harvest), and neuronal stages (5d after harvest) of differentiation (Figure 7.3 N, O).



7.3. SDF-1α Expression is Maintained Throughout the Stages of Neuronal Differentiation

A-D, E-H. Pluripotent control and SDF-1α cells expressed GFP and SDF-1α. **I-L, M-P.** Control and SDF-1α cells differentiated into neurons and retained GFP and SDF-1α expression. **N-O.** The sustained upregulation throughout the different stages of differentiation was confirmed with PCR. SDF-1α retained upregulation at the pluripotent stage, 1d and 5d after harvesting from neurospheres for terminal differentiation.

4. SDF-1α Upregulated Cells Secrete SDF-1α

SDF-1α and control cells were plated in 3cm dishes. The growth media was removed and replaced with 1ml of new growth media. The cells were allowed to secrete SDF-1α into the media for 8 hours and assayed with ELISA. This value was normalized against the live cells in the dish using the MTT assay. SDF-1α cells had a significantly greater concentration of SDF-1α compared to controls (n=8, p=0.043).

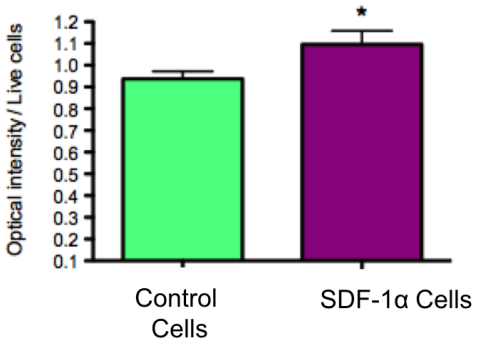


Figure 7.4. SDF-1α Upregulated Cells Secrete SDF-1α
To confirm that upregulated cells could secrete SDF-1α extracellularly, cell media was collected for ELISA assay after 8h of incubation in fresh media. The media was assayed for SDF-1α and normalized to cell density via MTT assay. SDF-1α upregulated cells had significantly more secretion compared to control cells (n=8, p=0.043)

5. SDF-1 Increases Migratory Capabilities In Vitro

As a proof of principle experiment, control and SDF-1α-upregulated HEK293 cells (Figure 7.5 A-D) were tested for migratory abilities with the wound-healing test after 24h in vitro. The wound-healing test illustrated that HEK293 cells with SDF-1α upregulation migrated to fill in the wound significantly more than HEK293 cells measured at 24 hours after initial scratch (Figure E-G, p=0.036). The effect of proliferation was controlled for with the inactivation of cells with mitomycin. Arrows show cell migration into the wound (Figure 7.5 F).

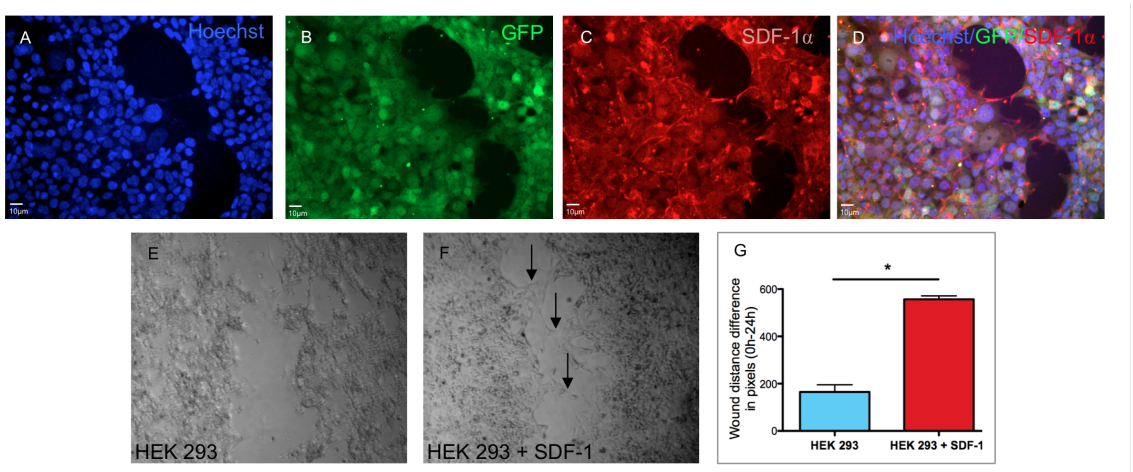


Figure 7.5. SDF-1 Increases Migratory Capabilities In Vitro
A-D. SDF-1α upregulated HEK293 cells expressed GFP and SDF-1α. **E-G.** The wound-healing test illustrates that HEK 293 cells with SDF-1α upregulation migrate to fill in the wound significantly more than HEK293 cells measured at 24 hours after initial scratch (p=0.036). Arrows show cell migration into the wound.

6. SDF-1α Increases Cell Survival

The cell survival of SDF-1α and control cells were tested with OGD insult for 0-7h. The cells were challenged with 0.1-0.3% oxygen in a hypoxia chamber and underwent a 12h reperfusion period afterwards. Cell survival was assayed with MTT cell viability assay as a normalizing control. Overall, cells from both control and SDF-1α cell groups responded to the OGD insult and fewer cells survived after longer durations of OGD. At 3h and 7h, there was significantly less cell survival in the control cells compared to the SDF-1α cells (n=6, p =0.001).

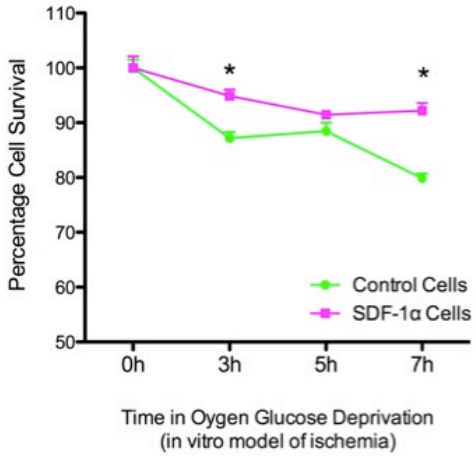


Figure 7.6. SDF-1α Increases Cell Survival
Cells were treated with OGD for 0-7h. Cell survival was assayed with MTT and normalized against control cells with no OGD. SDF-1α cells showed greater percentage of survival compared to control cells at 3h and 7h (n=6, p =0.001).

7. SDF-1α and Control Cells Differentiate into Functional Neurons In Vitro

SDF-1α cells and control cells both expressed the mature neuronal markers, neurofilament and NeuN after differentiation with the RA differentiation protocol (Figure 7.7 A-B, D-E). Further, the SDF-1α and control cells both exhibited action potentials with whole-cell patch clamp with inward sodium currents and outward potassium currents. TTX application diminished the inward sodium current (Figure 7.7 C, F)

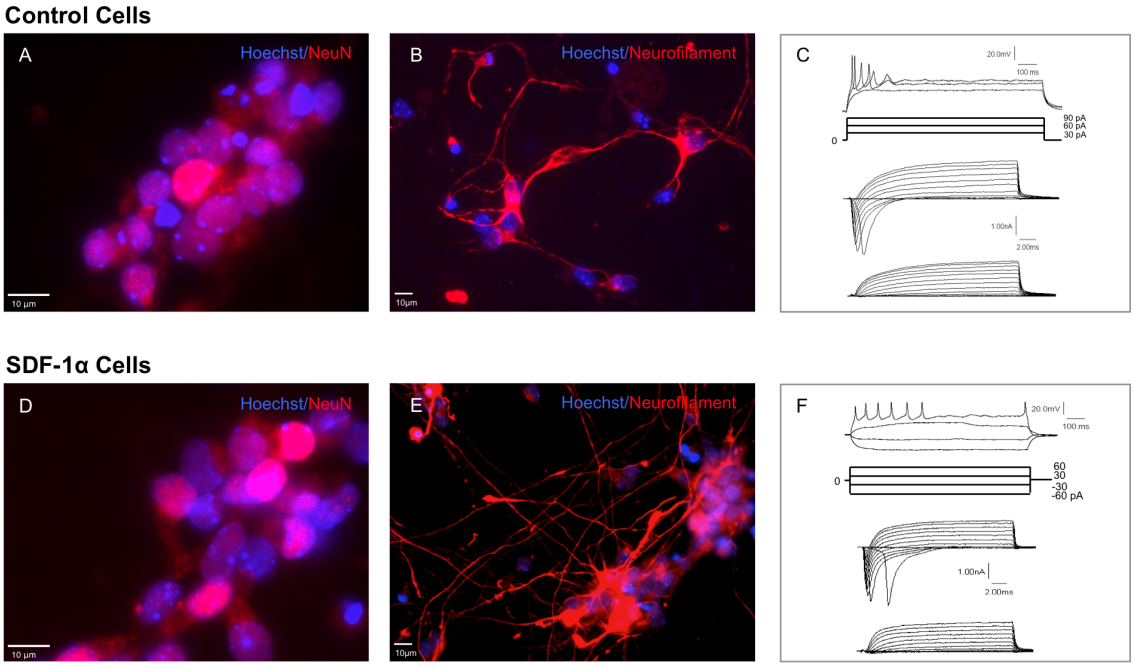


Figure 7. 7. SDF-1α and Control Cells Differentiate into Functional Neurons In Vitro
A-B, D-E. Control cells and SDF-1α cells differentiated into mature neurons expressing NeuN and neurofilament. **C, F.** Control cells and SDF-1α cells exhibited functional neuronal activity with action potentials, inward sodium current and outward potassium current. TTX application diminished the inward sodium current

8. SDF-1α Increases Differentiation

SDF-1α cells and control cells were differentiated using the RA differentiation protocol. Cells were harvested and plated for terminal differentiation on PDL/laminin-coated dishes and allowed to differentiate for 5d before immunocytochemistry. The cells were stained for nuclei (Hoechst) and NeuN. 6 photos were taken to sample several areas of the dishes from several passages. NeuN-positive cells were counted and divided by total number of Hoechst cells. SDF-1α cells had shown a significantly greater percentage of NeuN-positive cells compared to control cells (n=5-6, p= 0.0365).

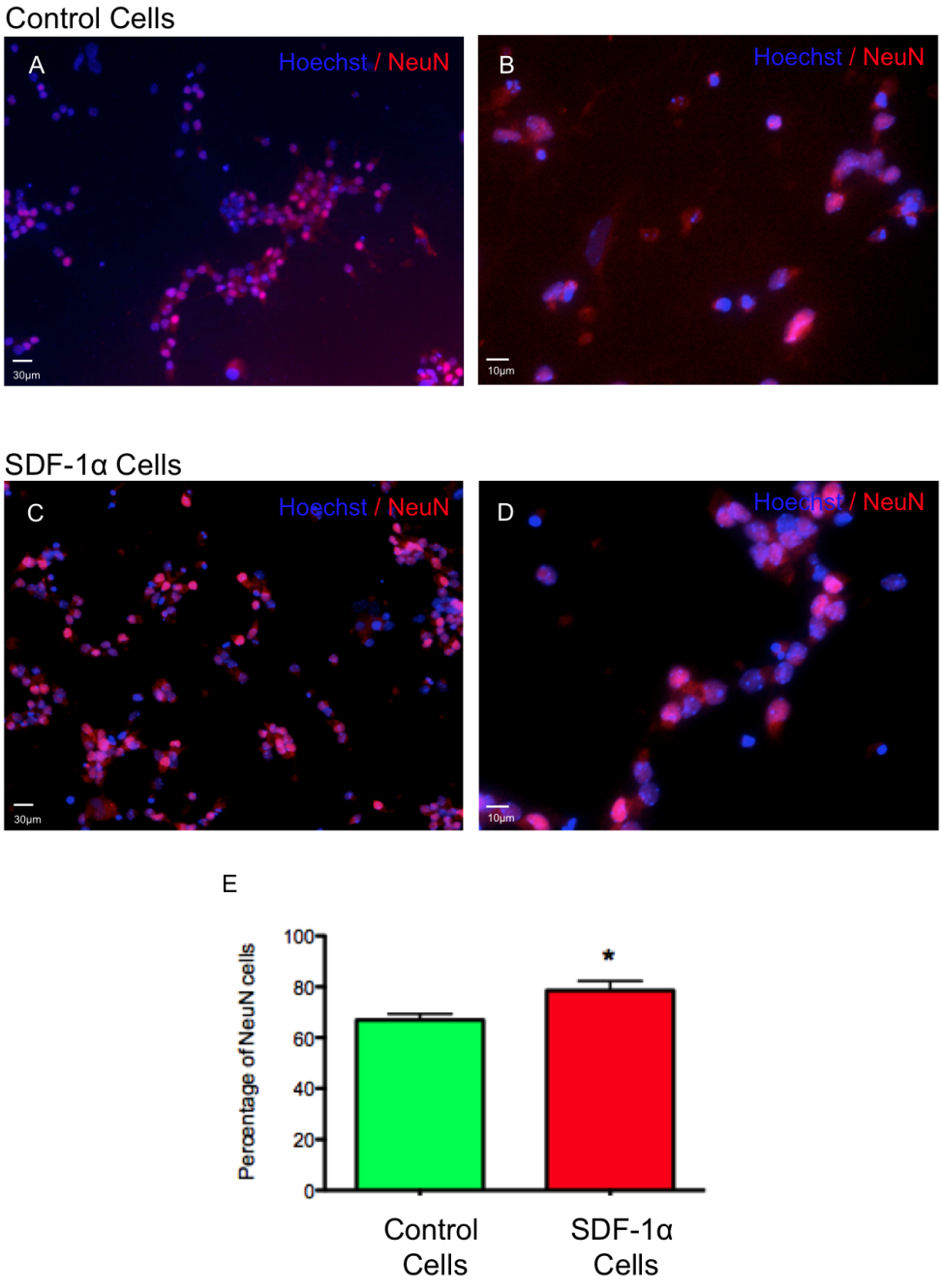


Figure 7. 8. SDF-1α Increases Differentiation
A-B, C-D. Control cells and SDF-1α cells differentiate into NeuN-positive cells at 5d after harvesting from neurospheres. **E.** When quantified against total number of Hoechst-labeled cells, SDF-1α upregulated cells showed a greater percentage of neuronal differentiation

C. Discussion

In the current investigation, we are the first to demonstrate the feasibility in creating a stable iPS cell line that retains SDF-1α upregulation throughout the different stages of neuronal differentiation. SDF-1α iPS cells are able to differentiate into functional neurons, increase cell survival, increase neuronal differentiation and secrete more SDF-1α. These findings provide the basis for using SDF-1α-upregulated iPS cells for transplantation therapy.

The ectopic upregulation of SDF-1α and CXCR4 have been used as tools to study chemotaxis in stroke and myocardial infarction, inflammation, and cancer metastasis[121,233,234]. Genetic upregulation in vitro and in vivo has been increasingly employed in basic research, translational, and clinical studies to understand gene function. One can specify a promoter in the plasmid construct to direct expression in a specific cell type such as using a GFAP promoter to drive astrocyte-specific expression of the gene of interest. Common constitutive promoters used in mammalian systems include cytomegalovirus (CMV), simian virus 40 (SV40), human ubiquitin C promoter (UBC), human elongation factor 1α (EF1α), phosphoglycerate kinase 1 (PGK), chicken β-actin coupled with CMV early enhancer (CAGG), murine stem cell virus (MSCV) long terminal repeat (LTR), and the gibbon ape leukgemia virus (GALV) LTR.

In the current study, we demonstrate the feasibility of using different promoters to upregulate SDF-1α in iPSCs. The levels of promoter-driven gene expression in differentiating pluripotent cells is important to examine since the expression can vary depending on the promoter and cell type employed[235,236]. For instance the EF1α promoter has demonstrated strong expression throughout all stages of ES cell differentiation

whereas CMV drives expression near the end stages of differentiation[235]. Contrastingly, MSCV LTR and the GALV LTR showed low expression at all stages of differentiation[235].

We had tested several methods to introduce plasmids into cells and eventually used lentiviral infection to create stable upregulation cell lines. Plasmids may be transfected directly using lipid-based methods, nucleofection, and electroporation. The efficiency of the transfection may be increased with the linearization of the plasmid to allow for easier integration into the host genome[237]. Plasmids may also be packaged for viral transduction in which the virus is applied directly into the cell media of the cells. The advantage of viral transduction is that the genetic material stably integrates into the host genome and daughter cells from transduced cells retain the gene of interest in their genome. Transduction is a useful method to create stable cell lines for laboratory testing. In this study, we utilize plasmids packaged into lentivirus to upregulate SDF-1α in iPS cells.

This chapter aims to characterize the upregulated cells in vitro preceding transplantation into a stroke model. First we were able to demonstrate that SDF-1α can be dramatically upregulated in iPSCs via plasmid constructs packaged into lentivirus. We tested several promoters to optimize expression and also considered the benefits and restraints of each. The CMV, CAG, EF1α promoters had been previously reported to have efficient expression in stem cells[238]. The EF1α promoter had been reported to drive expression at every stage of mouse ES cell differentiation whereas CMV promoter drove expression only at late stages of differentiation and showed robust differentiation after differentiation into neurons[236]. CMV showed less expression after neuronal differentiation[236]. CAG also was reported to drive expression significantly, but only in the

late stages of expression[235]. In our results, Lipofectamine was used to successfully transfect iPS cells with CMV, CAG, or EF1α promoter construct in which cells expressed the GFP reporter gene. We chose to utilize the EF1α promoter for its ability to drive expression at every stage of stem cell differentiation, its transfection efficiency when we tested the plasmid, and the puromycin selection factor present in the backbone of the plasmid allowing for a stable cell line creation.

EF1α stands for human elongation factor-1α, a constitutive promoter commonly used for ectopic gene upregulation in vitro and in vivo. EF1α has been reported to be one of the most successfully expressing promoters used in stem cells[238]. To confirm the upregulated expression of SDF-1α in cells transduced with SDF-1α virus, the RNA was extracted from the control and SDF-1α cell populations and PCR was run to examine SDF-1α expression. At the different volumes of SDF-1α virus application, there was a 5-6-fold increase of SDF-1α expression over the control virus cells of the same volume. Both the control and SDF-1α cells expressed GFP and SDF-1α and SDF-1α upregulated expression was maintained over the course of neuronal differentiation as demonstrated with PCR.

SDF-1α, like many trophic factors are secreted into the extracellular space. To support our hypothesis that SDF-1α-upregulated cells will encourage endogenous progenitor cell recruitment, we demonstrated that SDF-1α had a greater secretion from the SDF-1α-upregulated cells. Because stem cells naturally secrete trophic factors, we can expect that control iPS cells with no upregulation will also secrete and SDF-1α. In our observation when incubated in growth media for 8h, the cells with SDF-1α upregulation showed significantly more SDF-1α secretion in to the media compared to

control cells when normalized to living cells. The upregulation cell line has greater potential to recruit endogenous progenitors after transplantation compared to control cells.

Stem cell transplants can serve two roles, as trophic factor vehicles and as cell replacements. To demonstrate that control and upregulated stem cells have the potential to provide neuronal cell replacement, the cells underwent the RA differentiation protocol. They were differentiated into neurospheres and plated on PDL/laminin for terminal differentiation. The cells were stained for neuronal markers at 5d after harvest and whole-cell patch clamped 10d after harvest. The control and SDF-1α cell types expressed the mature neuronal marker, neurofilament at 5d after differentiation. 10d after differentiation, we tested their functionality after differentiation. Control and SDF-1α cells both exhibit action potentials and functional K^+ and Na^+ channels. In all, these histological and electrophysiological markers indicate that control and SDF-1α cells can be successfully differentiated into functional mature neurons displaying the potential for neuronal replacement after transplantation into the stroke brain. The neuronal differentiation into neurons is not impeded by SDF-1α upregulation in the iPSCs.

Another reason for SDF-1α upregulation was to bolster cell survivability since SDF-1α also plays a prominent role cell in cell survival[140]. Due to the cytotoxic nature of the stroke infarct, transplantation of exogenous cells has been challenging. A small percentage of cells survive after transplantation, thus justifying our upregulation of SDF-1α, a survival factor. To test this hypothesis, the cells were treated with an in vitro model of ischemia, OGD. When the cells were treated with the OGD insult, the SDF-1α cells had greater survival at 3 and 7h under hypoxic conditions. The SDF-1α released into the

media bound to the CXCR4 receptor on the cells to initiate the cascade of pathways that inhibits cell death through the inhibition of BAD, and also initiates the transcription pro-survival genes[140].

Thus far, few have investigated the role of SDF-1α on neuronal differentiation. GPCRs and trophic factors have been implicated in neuronal differentiation such as NGF through the Tropomyosin related kinase A (TrkA) signaling pathway[239,240]. SDF-1α has been shown previously to increase neuronal differentiation in pluripotent cells[241]. Upon the addition of several factors including SDF-1α, greater differentiation into dopaminergic neurons was observed[241]. When SDF-1α, secreted frizzled-related protein 1 (sFRP1), and vascular endothelial growth factor D (VEGFD) combined were added to the medium of NTera2 cells and hESC I6 cells, there was an increase in cells expressing TH and beta III tubulin[241]. Stromal factors help increase neuronal differentiation. Previously stromal cells mouse stromal cell line PA6 have been shown to genererate dopaminergic neurons from pluripotent stem cells[242], such as embryonic stem cells[243]. Stromal cells are a source of stromal cell-derived inducing activity (SCIA), the differentiation-inducing effect of stromal cells co-cultured with pluripotent cells[244]. This activity is due to secreted factors from stromal cells, one of these factors which is SDF-1[241]. Medium conditioned by the stromal cell line, PA6 has been shown to differentiate NTera2 cells and the hESC I6 cells into dopaminergic cells.

Here, we were the first to demonstrate that SDF-1α can be stably expressed in a stable cell line pluipotent cells, as neural progenitors 1d after harvest and as neurons 5d after harvest. SDF-1 α cells retain the ability to differentiate into neurons under the RA differentiation protocol despite their genetic manipulation. SDF-1α cells have greater

survivability under OGD conditions, and greater neuronal differentiation. These data demonstrate the potential for SDF-1α iPSCs to act as a vehicle for SDF-1α after transplantation into the brain as well as be able to provide neuronal cell replacement.

Chapter VIII

SDF-1α Upregulation in Transplanted iPSC-NPCs Increases Regeneration and Functional Recovery in an Ischemic Stroke Model

A. Introduction

Chemokines such as SDF-1α are highly upregulated in the infarct as an inflammatory and repair response after ischemic stroke[66]. SDF-1α plays pleiotropic roles in the brain after ischemic stroke. Upon ischemic occlusion, the HIF-1α system is activated when the tissues become hypoxic. SDF-1α is an adaptive downstream factor of HIF-1α. The result is the gradient expression of SDF-1α in infarct and peri-infarct area with SDF-1α expression strongest in the core[131-133]. Its expression peaks at 7 to 14 days after the insult and is produced by astrocytes and endothelial cells from the tissues surrounding the ischemic[134-136]. Once SDF-1α is secreted extracellularly, it can exert paracrine effects when it binds to its receptor, CXCR4 on other cells. One of these effects is the mobiliziation and homing of certain cell types when SDF-1α binds to CXCR4. As a chemokine, SDF-1α plays a role in cell chemotaxis in to many cell types.

CXCR4 is expressed in many progenitor cell types including neural progenitors[116], and endothelial progenitor cells[245]. Endogenously, SDF-1α mobilizes hematopoietic bone marrow stem cells that express CXCR4 to the peripheral blood and then to the stroke infarct for repair[110,137,144]. Rats injected with the SDF-1α protein 30 minutes after stroke revealed more BrdU-labeled engrafted cells in the ipsilateral cortex 3 days after stroke compared to the controls[137]. Similarly, GFP-positive bone marrow cells from a GFP transgenic rat injected into a donor revealed greater GFP cells in the penumbra after SDF-1α local injection compared to the controls[137]. With SDF-1α upregulation in the penumbra, neural stem cells transplanted into the contralateral hemisphere also migrate towards the site of ischemia[82]. These results suggest that SDF-

1α and its chemoattractive abilities for endogenous and exogenous stem cells provide a molecular target for a potential therapy for ischemic stroke.

This work focuses on the recruitment of endothelial progenitor cells and neural progenitors to the injury site for angiogenesis and neurogenesis. One source of neural progenitors is the SVZ. The SVZ is a niche of neural progenitors that migrate to the olfactory migratory stream under physiological conditions. Under stroke conditions, the neural progenitors are diverted from the rostral migratory stream to the injury area through the SDF-1α/CXCR4 mechanism of chemotaxis. Also under the SDF-1α/CXCR4 mechanism, endothelial progenitor cells that circulate in the vasculature are recruited to the ischemic injury leading to angiogenic repair. This migration of neural progenitors and endothelial cell progenitors to the injury area is the brain's attempt of self-repair. Unfortunately, these cellular events cannot fully repair the brain damage.

Another pleiotropic effect of SDF-1α is enhancing cell survival. This effect can be observed through the neuroprotective effects of SDF-1α in vitro and in vivo. SDF-1α increases cell survival through the expression of anti-apoptotic proteins. Cells exposed to hydrogen peroxide (H_2O_2) exhibited fewer caspase-3-positive cells and more Bcl-2 expression when SDF-1α was added in vitro[137]. Similarly, when SDF-1α protein was injected into the ischemic brain, infarct size decreased and motor function increased demonstrating that SDF-1α contributes to neuroprotection and repair in the brain[137].

SDF-1α has several beneficial effects in cell survival and progenitor cell recruitment thus in the current investigation, we utilize the benefits of SDF-1α in an overexpression paradigm in where we overexpress SDF-1α in transplanted iPS-NPCs. One major obstacle in stem cell transplantation is the low rate of cell survival after

transplantation. The upregulation of SDF-1α serves several benefits, primarily by increasing the cell survival of transplanted cells as well as releasing SDF-1α to supplement the endogenous levels of SDF-1α for tissue regeneration. We hypothesize that SDF-1α upregulation in transplanted iPSC-NPCs will increase the recruitment of progenitor cells to increase regeneration at the infarct region enhancing functional recovery after stroke.

B. Results

1. SDF-1α is Upregulated Endogenously After Stroke

Immunohistochemistry shows that SDF-1α is colabeled by GFAP-positive cells 7d after stroke in the peri-infarct area (Figure 8.1A-F). Western blot was used to probe for SDF-1 expression in the infarct core and the penumbra at 1, 3, and 7d after ischemic stroke. The tissue was dissected and homogenized to be run in a western blot. At 1, 3, and 7d after stroke SDF-1 expression was present. The core exhibited a greater SDF-1 expression compared to the stroke penumbra (Figure 8.1 G)

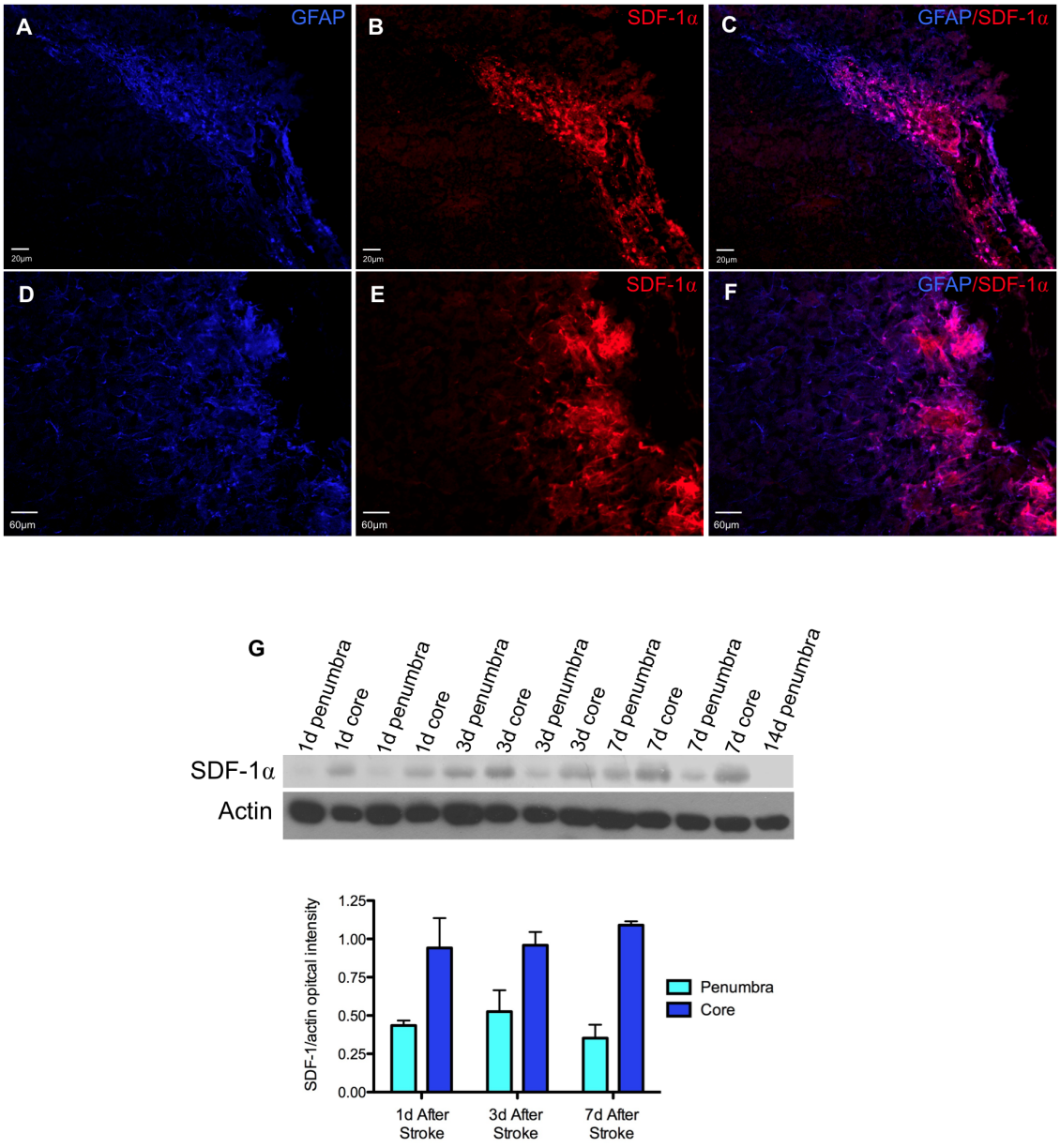


Figure. 8. 1. SDF-1α is Upregulated Endogenously After Stroke
A-C, D-F. SDF-1α and GFAP are expressed in the core and penumbra area 7d after the ischemic infarct. This is visualized at 20x and 40x. **G.** Western blot confirms the gradient nature of SDF-1α expression in the core and the penumbra at 1, 3, and 7 days after stroke.

2. Exogenous SDF-1α Increases Neurogenesis in the Peri-Infarct Area

Exogenous SDF-1α protein was injected into the peri-infarct cortex 7d after ischemic stroke. There were three groups in this experiment: sham, stroke only, and stroke + exogenous SDF-1α. Animals were injected with BrdU to label any cells undergoing S phase of mitosis starting 3d after stroke induction until sacrifice. Mice with stroke had a dramatic increase in BrdU cells in the peri-infarct region. Mice with stroke + SDF-1α showed an increasing trend in BrdU cells compared to stroke only animals. Similarly, when BrdU/ NeuN co-labeled cells were quantified, stroke mice had a greater number of BrdU/ NeuN cells compared to sham controls. Mice with stroke + SDF-1α showed an even greater number of BrdU/ NeuN cells compared to stroke only mice.

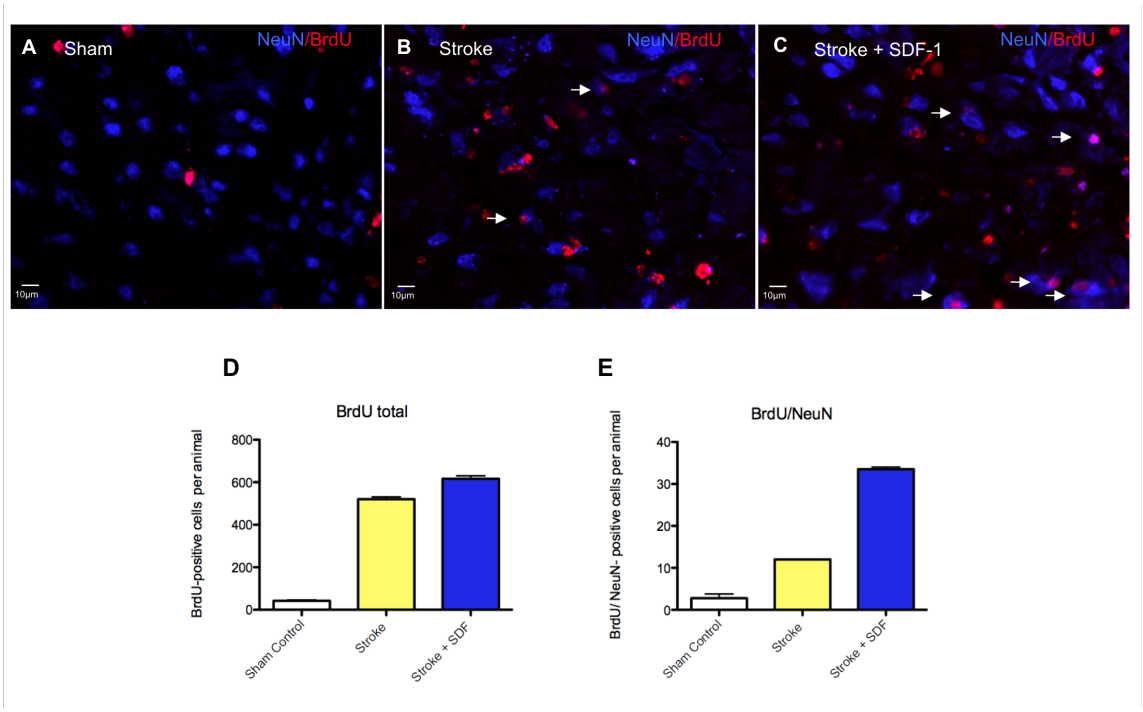


Figure 8.2. Exogenous SDF-1α Increases Neurogenesis in the Peri-Infarct Area
A-C. Stroke only animals exhibit increased BrdU and BrdU/NeuN co-label compared to sham. **D, E.** Stroke + SDF-1α show a trend towards more BrdU and BrdU/NeuN co-labeled cells compared to the stroke only group.

3. iPSC-NPCs With and Without SDF-1α Upregulation Exhibit Neuronal Differentiation In Vivo

Previously we demonstrated that iPSC-NPCs differentiated into neurons and glial cells after transplantation into the brain after ischemic stroke. To confirm that SDF-1α upregulated cells can still differentiate into neurons after transplantation, we transplanted control GFP and SDF-1α iPSC-NPCs into the adult mouse brain after 7d after stroke. Differentiating neurospheres were labeled with Hoechst at 1:10,000 concentration for 1h. The harvested iPSC-NPCs were transplanted into the peri-infarct cortex via local intracerebral injection. 300,000 cells suspended in 4μl of SATO was transplanted via 2 x 2μl injections at 2 sites in the peri-infarct cortex. We demonstrated that iPSC-NPCs derived from stable pluripotent GFP or GFP-SDF-1 cell lines can differentiate into NeuN-positive cells after transplantation. Figure 8.3 A, B, C demonstrate Hoechst/NeuN-positive cells after control cell transplantation visualized at 20x, 40x, and 100x magnification in the tissue 7d after transplantation. Figure 8.3 D, E, F demonstrate Hoechst/NeuN-positive cells after SDF-1α cell transplantation visualized at 20x, 40x, and 100x magnification in the tissue 7d after transplantation.

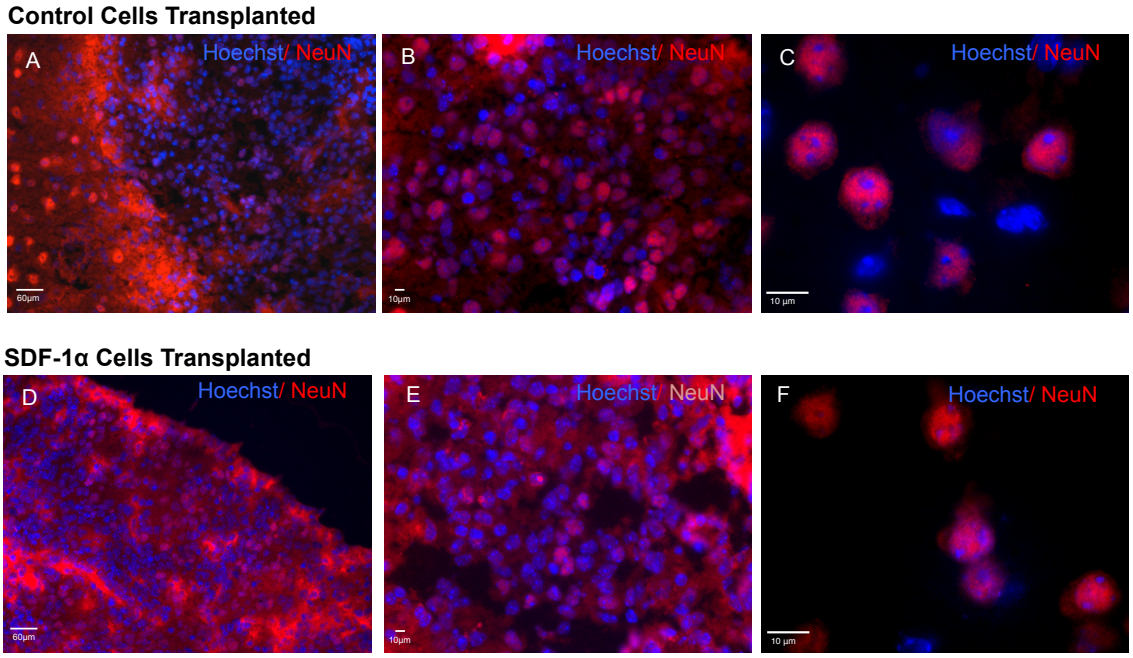


Figure 8.3. iPSC-NPCs With and Without SDF-1α Upregulation Exhibit Neuronal Differentiation In Vivo
A-C. Hoechst-transplanted control iPSC-NPCs co-labeled with NeuN visualized at 20x, 40x, and 100x magnification. **D-F.** Hoechst-transplanted SDF-1α iPSC-NPCs co-labeled with NeuN visualized at 20x, 40x, and 100x magnification.

4. Transplantation of SDF-1α iPSC-NPCs Increased Endogenous Angiogenesis

Dissected brain tissues were analyzed for neurogenesis and angiogenesis 14 days after transplantation. Animals were injected with BrdU (i.p.) on the first day of transplantation to label newly formed neuronal cells (NeuN-positive) and proliferating vessels (Glut-1-positive). In stroke only controls (Figure 8.4B) and iPSC-NPC transplanted brains (Figure 8.4C, D), we quantified the number of co-labeled NeuN/BrdU cells in the peri-infarct area (Figure 8.4 F) and co-labeled Glut-1/BrdU cells in the peri-infarct area (Figure 8 E). There were significantly more Glut-1/BrdU-positive cells (n=6-8; p=0.0142) in the SDF-1α iPSC-NPC transplantation group than in the stroke only group (Figure 8.4 E). There was a slight trend of increase toward the mice with transplantation

with NeuN/BrdU-positive cells (Figure 8.4 F). There were significant differences in neurogenesis when stroke, control iPSC-NPCs, and SDF-1α iPSC-NPCs were compared to sham (n=6-8; p= 0.0001, Figure 8.4 F).

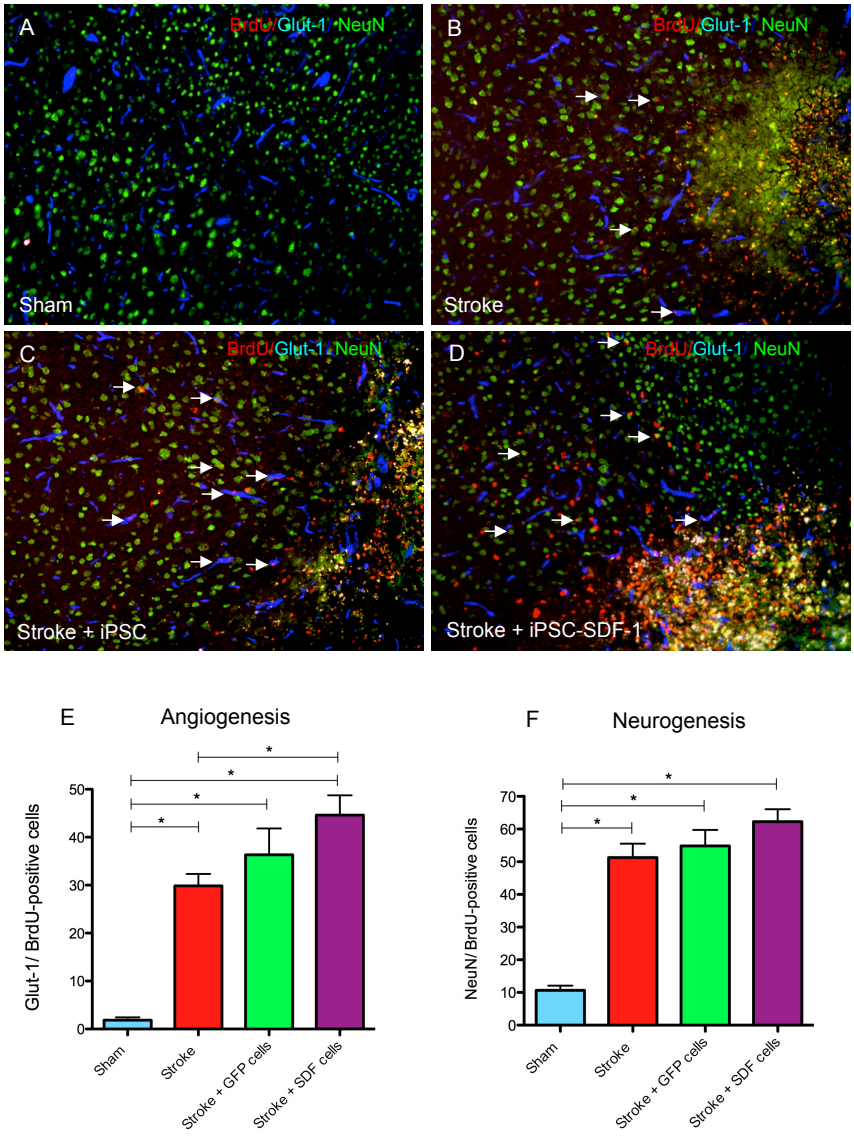


Figure 8.4. Transplantation of SDF-1α iPSC-NPCs Increased Endogenous Angiogenesis

A-D. Stroke only animals exhibit increased BrdU/NeuN and BrdU/Glut-1 co-label compared to sham. Stroke + control iPSC-NPC and Stroke + SDF-1α iPSC-NPC show an increase in BrdU/NeuN and BrdU/Glut-1 co-labeled cells compared to the stroke only group. **E.** There were significant increases in angiogenesis with SDF-1α iPSC-NPCs (n=6-8; p=0.0142) and **F.** Neurogenesis with all stroke groups compared to shams (n=6-8; p= 0.0001)

5. Transplantation of iPSCs In Vivo Increases Functional Recovery

Locomotor:

All the mice in the experimental groups were analyzed via a behavior tracking system, Top Scan from Clever Sys, Inc. The software recorded and analyzed the over overall distance migrated, time spent staying still, and velocity of movement in 1h in the testing chamber. Sham animals migrated an average distance of 26,240 mm over the course of 1h of the observation at 7d after stroke (Figure 8.5 C). Stroke only animals exhibited less movement migrating an average of 12,312.33 mm in 1h at 7d after stroke. There was significant difference in distance migrated with high dose of SDF-1 iPSC-NPC transplantation into stroke mice compared to stroke only controls at 7d (n=3-5, p=0.0202, Figure 8.5 C). The effects of the cell type transplantation on locomotor functional recovery were more pronounced 14d after transplantation compared to 7d. We also observed increases with control iPSC-NPC transplantations into stroke mice but significant greater increases in distance migrated with SDF-1α iPSC-NPC transplantation. Low and high dose groups of SDF-1α iPSC-NPC transplanted mice both showed greater migration over stroke only controls. Low and high dose SDF-1α iPSC-NPC transplanted mice both exhibited significantly greater migration over low dose control iPSC-NPC transplanted mice at 14d after stroke (n=3-5, p=0.0021, Figure 8.5 F) .

In the measurements for the time animals spent standing still in the 1h observation period (Figure 8.5 D), at 7d we observed an inverse trend in the experimental groups compared to distance migrated. Experimental groups with greater migration distances showed less time spent staying still. There were no significant differences at 7d, but we observe significant differences at 14d after transplantation (Figure 8.5 G). High SDF-1α

iPSC-NPC transplantation groups showed significant differences of less time stayed still compared to stroke only animals and low dose control iPSC-NPC transplantation mice at 14d after transplantation.

The velocity was also measured in the same 1h observation period at 7d and 14d after iPSC-NPC transplantation. We observed significant differences between high SDF-1α iPSC-NPC transplantation mice and low control iPSC-NPC transplantation mice, and stroke only controls 7d after transplantation ($n=3\text{-}5$, $p=0.0129$). The effect of iPSC-NPC transplantation and the upregulation of SDF-1α is more pronounced at 14d after transplantation. We observed significant differences ($n=3\text{-}5$, $p=0.002$) between low SDF-1α iPSC-NPC transplantation group and stroke only group, high SDF-1α iPSC-NPC transplantation group and stroke only group, high SDF-1α iPSC-NPC transplantation groups and low control iPSC-NPC transplantation groups, low SDF-1α iPSC-NPC transplantation group, and low SDF-1α iPSC-NPC transplantation group.

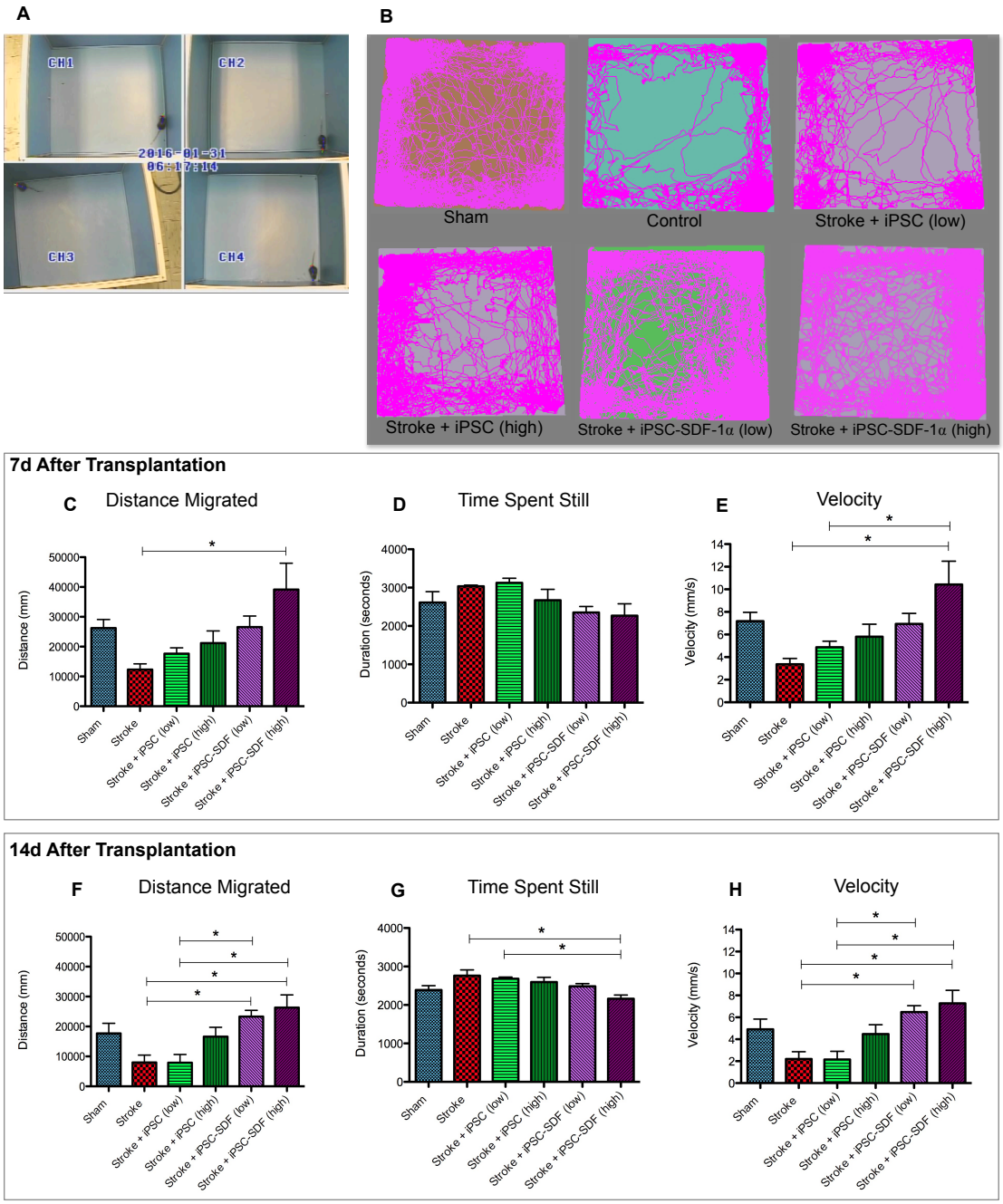


Figure 8. 5. Transplantation of iPSCs In Vivo Increases Functional Recovery: Locomotion

A. Testing chambers recorded with TopScan analysis system. **B.** Representative migration traces from sham, stroke only, and stroke + transplantation groups. **C.** 7d after transplantation, control iPSC-NPC transplantation at low and high doses showed trends of more migration compared to stroke only mice. The high dose SDF-1α iPSC-NPC transplantation group showed a significant difference compared to stroke only mice **D.** Experimental groups with greater migration distances showed a trend towards less time spent staying still at 7d. **E.** We observed significant differences between high SDF-1α

iPSC-NPC transplantation mice and low control iPSC-NPC transplantation mice, and stroke only controls 7d after transplantation (n=3-5, p=0.0129) **F.** Low and high dose SDF-1α iPSC-NPC transplanted mice both exhibited significantly greater migration over low dose control iPSC-NPC transplanted mice at 14d after stroke **G.** High SDF-1α iPSC-NPC transplantation groups showed significant differences of less time stayed still compared to stroke only animals and low dose control iPSC-NPC transplantation mice at 14d after transplantation. **H.** We observed significant differences between low SDF-1α iPSC-NPC transplantation group and stroke only group, high SDF-1α iPSC-NPC transplantation group and stroke only group, high SDF-1α iPSC-NPC transplantation groups and low control iPSC-NPC transplantation groups, low SDF-1α iPSC-NPC transplantation group and low SDF-1α iPSC-NPC transplantation group (n=3-5, p=0.002).

Sensorimotor:

We analyzed the somatosensory function of sham, stroke, control iPSC-NPC, and SDF-1α iPSC-NPC transplantation animals using the vibrissae-elicited forelimb placement test 7 and 14 days after transplantation. An adhesive sticker is placed on the right or left paw at random for 4 trials. Mice were trained and acclimated to the adhesive removal test equally to all treatment groups at least one day before testing. The time-to-notice the sticker and time-to-remove sticker are recorded (in seconds). The animal will take more time to notice and remove the sticker from the deficient contralateral paw compared to the ipsilateral paw. The 4 trials for the left and right paw notice and removal were averaged. Data was analyzed with completely randomized two-way ANOVA.

Figures 8.6 A, B illustrate the time for the mouse to notice the sticker once it was placed on its paw. This is indicated by a shaking of that paw or a sniffing. Sham animals with no deficit on either side exhibited similar times to notice for both right and left paws at 7d after transplantation. Their times to notice were also lower on the left side compared to all other experimental stroke and stroke + iPSC-NPC groups (Figure 8.6 A). The stroke only control group, the left deficient side showed a higher trend of the mouse

taking longer to notice the sticker on the left side. There was a trend of reduction in time to remove for the left side in animals that received low and high doses of control iPSC-NPC and SDF-1α iPSC-NPC (Figure 8.6 A). The time to notice on the right side stayed consistent in all experimental groups at 7d after transplantation at or around 2 seconds.

At 14d after transplantation, the time to notice for sham animals were similar times to notice for both right and left paws. Their times to notice were also lower on the left side compared to all other experimental stroke and stroke + iPSC-NPC groups (Figure 8.6 B). At 14d after stroke, the differences between the stroke only group and the transplantation groups' time to notice the sticker evened out to around 4 seconds to notice. There were no differences observed in the left side between stroke only group and iPSC-NPC transplantation groups (Figure 8.6 B). The time to notice on the left side of the stroke and transplantaiton groups still remained higher than the right side paw time to notice.

The time to remove reflects the motor abilities of the mouse. We examine the times to remove 7d (Figure 8.6C) and 14d (Figure 8.6D) after transplantation. Similar to the trends observed in time to notice, we observed that the sham animals at 7d exhibited the lowest times to remove for both the left and the right sides (Figure 8.6C). In the stroke only animals, the left side time to remove showed a greater time to removed compared to sham animals and iPSC-NPC transplantation animals. Stroke animals with high and low doses of control and SDF-1 iPSC-NPC transplantation had lower right side times to remove compared to stroke only animals, however there were no differences between the control and SDF-1 iPSC-NPC cell types at 7d after transplantation.

In examining the time to remove the sticker 14d after transplantation (Figure 8.6D), sham animals exhibited similar removal times with right and left paw. There were no differences observed in the left side between stroke only group and iPSC-NPC transplantation groups (Figure 8.6 D). The time to remove on the left side of the stroke and transplantation groups still remained higher than the right side paw time to notice.

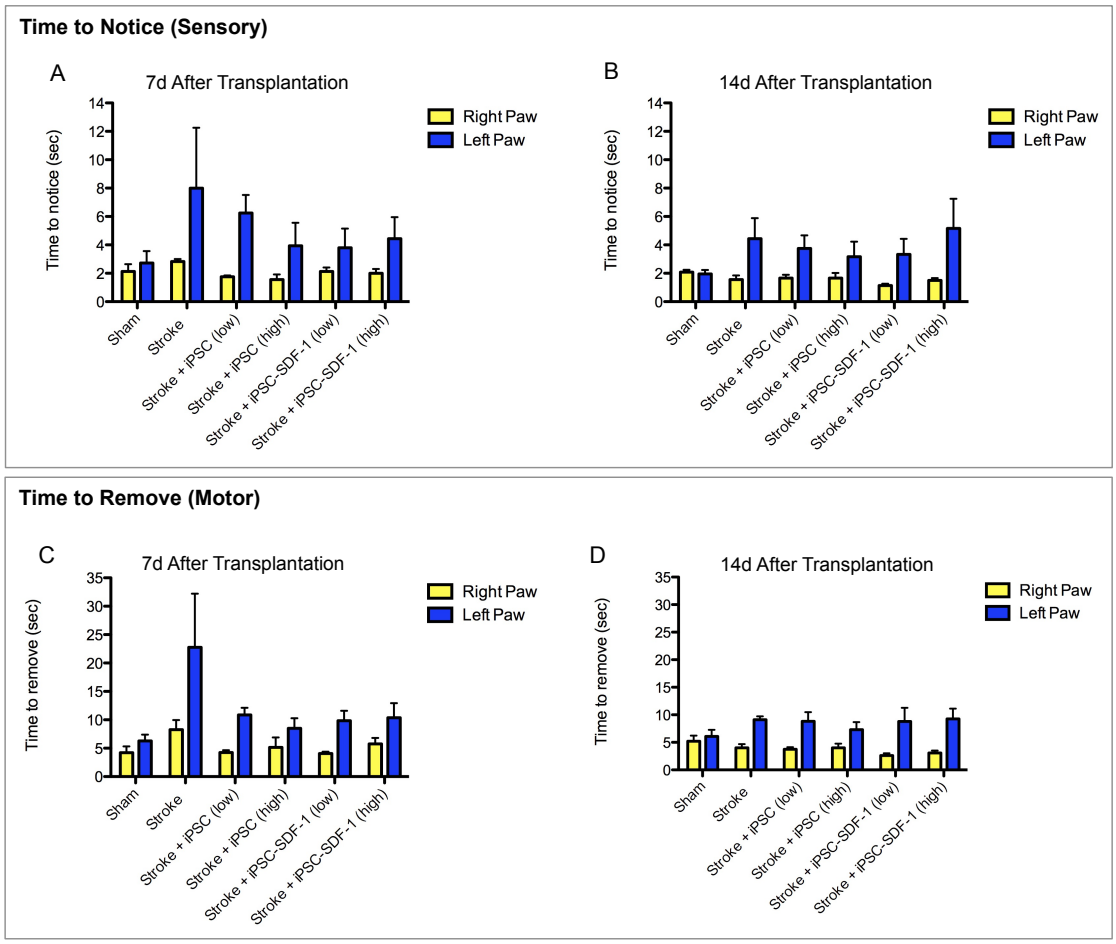


Figure 8.6. Transplantation of iPSCs In Vivo Increases Functional Recovery: Sensorimotor

A. At 7d after transplantation, stroke only mice showed a longer time to notice compared to cell transplantation mice. Control iPSC-NPC and SDF-1α iPSC-NPC transplantationgroups showed a trend of reduced times to notice compared to stroke. **B.** The differences between stroke only and transplantation groups were not apparent 14d after transplantation, however stroke groups' time to notice remained higher than sham times. **C.** At 7d after transplantation, stroke only mice had a longer time to remove

compared to cell transplantation mice. Similar to time-to-notice data, control iPSC-NPC and SDF-1α iPSC-NPC transplantation groups showed a trend of reduced times to remove compared to stroke only. **D.** The differences between stroke only and transplantation groups were attenuated by 14d after transplantation, however stroke groups' time to remove remained higher than sham times.

C. Discussion

In the previous chapter of this dissertation, we demonstrated the feasibility of stable SDF-1α upregulation in iPSCs. The SDF-1α-upregulated cells could be differentiated into functional neurons and also could release SDF-1α extracellularly. Our next objective was to test the cells as a therapeutic by transplanting them into a model of focal ischemic stroke. We hypothesized that there would be greater regeneration and functional recovery in stroke animals that received transplantations of iPSC-NPCs with and without SDF-1α upregulation.

To demonstrate that our focal ischemic stroke model was consistent with the literature in which SDF-1α was expressed in the infarct and penumbra after stroke, we performed immunohistochemistry on tissues 7d after stroke for GFAP and SDF-1α. We observed that there were more active astrocytes in the peri-infarct region 7d after stroke compared to non-infarcted tissue. We also observed that much of the SDF-1α expression was co-labeled with the astrocyte stain. This is consistent with the literature that reactive astrocytes are one source of SDF-1α expression after ischemic stroke[80,135]. Another source is from vessel cells in the peri-infarct region[246]. Vessels expressing SDF-1α have been reported to be scaffolds for neuroblasts to migrate to the injury[246].

To further confirm the dynamic upregulation of SDF-1α in our ischemic stroke model, we ran a western blot for the dissected tissue from the core and tissues of our

stroke animal model. We expected to see SDF-1α expressed in a gradient fashion[66]. Our western blot results showed high expression in the core and lower expression in the penumbra at 1, 3, and 7d after stroke. This informs our time line for cell transplantation. Our transplantations were performed in the delayed phase of stroke at 7d to avoid the cytotoxic milieu of the acute phase of stroke. We wanted to confirm with western blotting that SDF-1α was expressed 7d after stroke on the day of transplantation in order to supplement the endogenous SDF-1α through iPSC-NPC transplantation.

As a proof of principle, we demonstrate that a one-time injection of 600ng of SDF-1α protein into the peri-infarct area of mice at 7d after stroke increased the number of BrdU cells and BrdU/NeuN co-labeled cells in the peri-infarct area. This illustrated a trend of greater neurogenesis in mice with exogenous SDF-1α protein injection compared to shams and stroke only mice. This is consistent with previous work using exogenous SDF-1α injection into the brain leading to greater neurogenesis at the infarct[132]. Not all the BrdU-positive cells were NeuN co-labeled however, suggesting that some BrdU-labeled cells were labeling different cell types including endothelial progenitor cells, glia and microglia[80,246,247]. Since SDF-1 is also a chemokine, it can also attract immune cells such as lymphocytes and monocytes as well as regenerative progenitors reflecting the functional complexity of SDF-1[109]. The roles of SDF-1 are dichotomous in that it perpetuates inflammation in the acute phase of stroke, and encourages regeneration in the delayed phase of stroke[134]. This may be modulated by isoform-specific SDF-1 expression. The SDF-1 peptide has several splice variants including SDF-1α and SDF-1β. SDF-1β is not as abundantly expressed as SDF-1α and is upregulated in endothelial cells after stroke. Evidence suggests that SDF-1β plays a greater role as a chemoattractant for

peripheral blood cells such as ciruculating leukocytes into the ischemic area whereas SDF-1α has more of an effect on neuronal plasticity after stroke[134,248]. This is an example of the elegance and complexity of how one gene can modulate several events after an injury.

To support our hypothesis that iPSC-NPCs both with and without SDF-1α upregulation can have the potential for neuronal cell replacement, we transplanted the iPSC-NPCs in to the peri-infarct area. The cells stained positive for mature neuronal marker after transplantation. This represents the first step of neuronal cell replacement establishing that the iPSC-NPCs can differentiate in vivo. The RA differentiation protocol differentiates the cells into cortical forebrain neurons[169,209,249]. Previous work in our lab has confirmed that the cells stain positively for neurofilament, synapsin, SNAP25, FOXG1, a forebrain marker, Dopamine Beta Hydroxylase (DBH), the noradrenergic marker, and express trophic factors such as VEGF and BDNF. Several groups have demonstrated that transplanted pluripotent stem cells, ES[158,185] or iPS[158,172] cells have differentiated into neurons after engraftment. However, further studies are needed to understand the long-distance connections that engrafted cells establish. In one such study, primate embryonic stem cell-derived neural progenitor cells (ESC-NPCs) were transplanted into mice and the retrograde tracer, fluorogold was used to label any long-distant connections that the engrafted cells made[161]. By 28d after engraftment, many of the engrafted cells expressed synaptophysin. Fluorogold-labeled cells were found in the substantia nigra and the anterior thalamus 14 and 28d after transplantation into the striatum[161]. Another group was able to demonstrate functional cortico-thalamic integration into the host circuitry from the neural stem cells engrafted into the rat

cortex[250]. They used fluorogold to trace axonal connections of the engrafted cells to their targets. They demonstrated that the engrafted cells could receive excitatory synaptic inputs from host neurons by electrically stimulating the surrounding cortex to evoke excitatory postsynaptic potentials (ESPS) in the engrafted cells[250]. The field of exogenous cell transplantation currently lies at the forefront of re-establishing circuitry after injury.

To support our hypothesis that the transplantation of iPSC-NPCs and iPSC-NPCs with SDF-1α upregulation will enhance the recruitment of progenitor cells, we demonstrate an increase in angiogenesis with SDF-1α iPSC-NPC transplantation. We observed a trend in neurogenesis and expected a greater effect of SDF-1α and transplantation on the recruitment of neural progenitors. We had previously observed a neurogenic effect with iPSC-NPC transplantation in neonatal rats in Chapter 6. The difference in neurogenesis could be due to the different ischemic stroke models, one being neonatal and the other in adult rodents. The increase in proliferation was already occurring endogenously in neonatal rats to encourage more regeneration after stroke, whereas the adult mice did not have a high basal level of proliferation during stroke. It is possible that the concentration of SDF-1α released into the tissue from the cells was not sufficient to cause a more remarkable effect in the endogenous neural progenitors. Furthermore, the peri-infarct tissue lies at a closer proximity to endothelial progenitor cells. Endothelial progenitor cells are hematopoietic in origin and disseminate in the body via the circulatory system[251]. The endothelial progenitor cells have closer access to the infarct whereas neural progenitors must migrate from the SVZ. It is possible that with more time after the iPSC-NPC transplantation, we would eventually observe greater total neural progenitor migration to the peri-infarct area.

We analyzed two modes of functional recovery of the mice after the transplantation of iPSC-NPCs: animal locomotion/ overall movement, and sensorimotor function. We demonstrated that animals with iPSC-NPCs transplantation and SDF-1α iPSC-NPC transplantation had significantly more migration overall compared to stroke only animals at 14d after transplantation. The transplanted animals SDF-1α iPSC-NPC also had a significantly greater velocity in their movement compared to stroke only animals and control iPSC-NPC transplantation groups. Based on the angiogenesis and trend in neurogenesis observed with SDF-1α iPSC-NPC transplanted mice, the improved functional recovery could be due to neurovascular coupling in the peri-infarct area[80]. Angiogenesis represents a histological correlate to the improved functional recovery by providing new blood flow as support to the injured cells in the peri-infarct area.

The adhesive removal was performed to test the somatosensory area affected by the infarct. Our stroke model targets the whisker barrel cortex specifically, but also affects the adjacent forepaw somatosensory area as well. This model thus lends itself to be tested via the adhesive removal test, a sensorimotor test. At 7d, but not 14d after transplantation, all groups with transplantation showed a trend towards a quicker time to notice and remove the sticker compared to stroke only group. The effect of transplantation had diminished through a learning/plasticity effect by 14d after transplantation. We have observed this recovery effect consistently in our lab with this stroke model.

With SDF-1α iPSC-NPC transplantation, we observed an effect on locomotion, but not a significant effect on sensorimotor function. This improvement on locomotion and overall migration may be explained by SDF-1α playing a role in modulating the

firing patterns of neurons[252-254]. Currently, there has been little research on the effect of SDF-1α on neuronal function, however there are some reports demonstrating SDF-1α modulates the release arginine vasopressin (AVP) in the magnocellular neurons of the supraoptic nucleus (SON) and the paraventricular hypothalamic nucleus. The changes in AVP release could initiate a systemic effect affecting overall movement and health. Another report demonstrates that SDF-1 enhances GABA and glutamate synaptic activity at serotonergic neurons modulating serotonin release[254]. This may be a systemic neurotransmitter correlate to explain improved locomotion.

Collectively, this data demonstrates that iPSC-NPC transplantation enhances angiogenesis and locomotor functional recovery after ischemic stroke. Furthermore, the upregulation of SDF-1α in iPSC-NPC further contributed angiogenesis and functional recovery after ischemic stroke. Such genetic manipulations to enhance the transplantation of iPSC-NPC and regeneration is an important tool for regenerative therapy.

Chapter IX
Summary and Conclusions

Ischemic stroke is a far-reaching and devastating disease. It is the fourth leading cause of death behind heart disease, cancer, and chronic lower respiratory disease (CLRD)[1]. Every year in the U.S., 795, 000 people experience a new or recurrent stroke[1]. About 1 of 19 deaths are associated with stroke. 87% of strokes are ischemic in nature. It remains a great financial burden to our healthcare system costing about $70 billion to Americans each year.

Despite its prevalence, there is only one FDA-approved treatment for ischemic stroke, tPA. tPA is a thrombolytic drug that dissolves the clot allowing the blood flow to return. However, the use of tPA is very time-sensitive and must be administered 4.5h after a stroke occurs[5]. The time of stroke may be difficult to pinpoint especially if the person was asleep during the occurrence. This and other medical criteria preclude the vast majority of stroke sufferers from actually received tPA. Only less than 5% of people end up receiving the drug[8]. Therefore, those that receive tPA have a greater risk of functional deficits and permanent tissue damage. In this dissertation, we address the need for regenerative therapies after stroke.

One avenue for regenerative therapies is the use of stem cell transplantation for the regeneration of brain tissue. A novel cell type created by Yamanaka in 2007 is a new source of pluripotent cells. These cells can be created from one's own adult somatic cells allowing for an autologous transplantation avoiding post-transplantation complications with immune rejection. Further, this cell type circumvents the controversy surrounding obtaining stem cells from pluripotent cells.

In this dissertation, first we have shown that the transplantation of iPSC-NPCs can enhance the endogenous regeneration that occurs after stroke via the release of

trophic factors into the tissue. iPSC-NPC transplantation also have the advantage of providing more neuronal cells into the injury area. Peri-natal rats with this iPSC-NPC transplantation exhibited greater neurogenesis and angiogenesis at the peri-infarct area. Further, they showed functional improvements 14d after the transplantation. At this highly proliferative and regenerative age, we were able to encourage more regenerative to this area after ischemic stroke and see behavioral improvements.

Because trophic factors play an essential role to endogenous regeneration, our goal was to upregulate one of these essential factors, SDF-1α. SDF-1α is a potent chemoattractant upregulated in the stroke infarct. SDF-1α recruits cells to the infarct such as endothelial progenitor cells and neural progenitor cells for regeneration. SDF-1α also increases the cell survival of cells allowing for more cells to survive after transplantation. We have demonstrated that transplantation of SDF-1α iPSC-NPCs after stroke increases angiogenesis and functional recovery.

These results give hope to the use of iPSC-NPCs for the regenerative treatment of ischemic stroke. Further work would need to be done in order to be able to translate this work into a human therapy. The optimization of cell dosage must be explored and the tumorigenicity of the cells needs to be vigorously tested before clinical transplantation. By using trophic factor-enhanced iPSC-NPCs we provide evidence that we may be able to create a regenerative therapy for those affected by stroke and other neurodegenerative disorders.

Chapter X
References

References:

1 Go, AS, Mozaffarian, D, Roger, VL *et al*. Heart Disease and Stroke Statistics--2013 Update: A Report From the American Heart Association. *Circulation* **127**, e6-e245, doi:10.1161/CIR.0b013e31828124ad (2013).

2 Centers for Disease, C & Prevention. Prevalence of stroke--United States, 2006-2010. *MMWR. Morbidity and mortality weekly report* **61**, 379-382 (2012).

3 Howard, G, Anderson, R, Johnson, NJ *et al*. Evaluation of social status as a contributing factor to the stroke belt region of the United States. *Stroke* **28**, 936-940 (1997).

4 Heidenreich, PA, Trogdon, JG, Khavjou, OA *et al*. Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. *Circulation* **123**, 933-944, doi:10.1161/CIR.0b013e31820a55f5 (2011).

5 Zoppo, D. Expansion of the Time Window for Treatment of Acute Ischemic Stroke With Intravenous Tissue Plasminogen Activator: A Science Advisory From the American Heart Association/American Stroke Association (vol 40, pg 2945, 2009). *Stroke* **41**, E562-E562, doi:Doi 10.1161/Str.0b013e3181efa8d7 (2010).

6 Wahlgren, N, Ahmed, N, Davalos, A *et al*. Thrombolysis with alteplase 3-4.5 h after acute ischaemic stroke (SITS-ISTR): an observational study. *Lancet* **372**, 1303-1309, doi:10.1016/S0140-6736(08)61339-2 (2008).

7 Graham, GD. Tissue plasminogen activator for acute ischemic stroke in clinical practice - A meta-analysis of safety data. *Stroke* **34**, 2847-2850, doi:Doi 10.1161/01.Str.0000101752.23813.C3 (2003).

8 Adeoye, O, Hornung, R, Khatri, P *et al*. Recombinant tissue-type plasminogen activator use for ischemic stroke in the United States: a doubling of treatment rates over the course of 5 years. *Stroke* **42**, 1952-1955, doi:10.1161/STROKEAHA.110.612358 (2011).

9 Kleindorfer, D, Kissela, B, Schneider, A *et al*. Eligibility for recombinant tissue plasminogen activator in acute ischemic stroke: a population-based study. *Stroke* **35**, e27-29, doi:10.1161/01.STR.0000109767.11426.17 (2004).

10 Pancioli, AM, Broderick, J, Kothari, R *et al*. Public perception of stroke warning signs and knowledge of potential risk factors. *JAMA : the journal of the American Medical Association* **279**, 1288-1292 (1998).

11 Schroeder, EB, Rosamond, WD, Morris, DL *et al*. Determinants of use of emergency medical services in a population with stroke symptoms: the Second Delay in Accessing Stroke Healthcare (DASH II) Study. *Stroke* **31**, 2591-2596 (2000).

12 Reeves, MJ, Arora, S, Broderick, JP *et al*. Acute stroke care in the US: results from 4 pilot prototypes of the Paul Coverdell National Acute Stroke Registry. *Stroke* **36**, 1232-1240, doi:10.1161/01.STR.0000165902.18021.5b (2005).

13 Green, AR. Protecting the brain: the search for a clinically effective neuroprotective drug for stroke. *Critical reviews in neurobiology* **16**, 91-97 (2004).

14 Marler, JR. NINDS clinical trials in stroke: lessons learned and future directions. *Stroke* **38**, 3302-3307, doi:10.1161/STROKEAHA.107.485144 (2007).

15 Chau, M, Chen, D & Wei, L. Erythropoietin attenuates inflammatory factors and cell death in neonatal rats with intracerebral hemorrhage. *Acta Neurochir Suppl* **111**, 299-305, doi:10.1007/978-3-7091-0693-8_50 (2011).

16 Ehrenreich, H, Weissenborn, K, Prange, H *et al*. Recombinant human erythropoietin in the treatment of acute ischemic stroke. *Stroke* **40**, e647-656, doi:10.1161/STROKEAHA.109.564872 (2009).

17 Lee, J-M, Grabb, MC, Zipfel, GJ *et al*. Brain tissue responses to ischemia. *The Journal of Clinical Investigation* **106**, 723-731 (2000).

18 Brown, AM & Ransom, BR. Astrocyte glycogen and brain energy metabolism. *Glia* **55**, 1263-1271, doi:10.1002/glia.20557 (2007).

19 Astrup, J, Symon, L, Branston, NM *et al*. Cortical evoked potential and extracellular K+ and H+ at critical levels of brain ischemia. *Stroke* **8**, 51-57 (1977).

20 Barone, FC & Feuerstein, GZ. Inflammatory mediators and stroke: new opportunities for novel therapeutics. *J Cereb Blood Flow Metab* **19**, 819-834, doi:10.1097/00004647-199908000-00001 (1999).

21 Proskuryakov, SY, Konoplyannikov, AG & Gabai, VL. Necrosis: a specific form of programmed cell death? *Exp Cell Res* **283**, 1-16 (2003).

22 Choi, DW. Excitotoxic cell death. *J Neurobiol* **23**, 1261-1276, doi:10.1002/neu.480230915 (1992).

23 Choi, DW. Ischemia-induced neuronal apoptosis. *Curr Opin Neurobiol* **6**, 667-672 (1996).

24 Niquet, J, Baldwin, RA, Allen, SG *et al*. Hypoxic neuronal necrosis: protein synthesis-independent activation of a cell death program. *Proc Natl Acad Sci U S A* **100**, 2825-2830, doi:10.1073/pnas.0530113100 (2003).

25 Banasiak, KJ, Xia, Y & Haddad, GG. Mechanisms underlying hypoxia-induced neuronal apoptosis. *Prog Neurobiol* **62**, 215-249 (2000).

26 Kerr, JF, Wyllie, AH & Currie, AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-257 (1972).

27 Fadok, VA, Voelker, DR, Campbell, PA *et al*. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* **148**, 2207-2216 (1992).

28 Martin, SJ, Reutelingsperger, CP, McGahon, AJ *et al*. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med* **182**, 1545-1556 (1995).

29 Fadok, VA, Savill, JS, Haslett, C *et al*. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J Immunol* **149**, 4029-4035 (1992).

30 Bortner, CD, Oldenburg, NB & Cidlowski, JA. The role of DNA fragmentation in apoptosis. *Trends in cell biology* **5**, 21-26 (1995).

31 Walker, PR, Smith, C, Youdale, T *et al*. Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res* **51**, 1078-1085 (1991).

32 Collins, RJ, Harmon, BV, Gobe, GC *et al*. Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. *International journal of radiation biology* **61**, 451-453 (1992).

33 Enright, H, Hebbel, RP & Nath, KA. Internucleosomal cleavage of DNA as the sole criterion for apoptosis may be artifactual. *The Journal of laboratory and clinical medicine* **124**, 63-68 (1994).

34 Peitsch, MC, Muller, C & Tschopp, J. DNA fragmentation during apoptosis is caused by frequent single-strand cuts. *Nucleic acids research* **21**, 4206-4209 (1993).

35 Broughton, BR, Reutens, DC & Sobey, CG. Apoptotic mechanisms after cerebral ischemia. *Stroke* **40**, e331-339, doi:10.1161/STROKEAHA.108.531632 (2009).

36 Gao, Y, Liang, W, Hu, X *et al*. Neuroprotection against hypoxic-ischemic brain injury by inhibiting the apoptotic protease activating factor-1 pathway. *Stroke* **41**, 166-172, doi:STROKEAHA.109.561852 [pii] 10.1161/STROKEAHA.109.561852.

37 Ankarcrona, M, Dypbukt, JM, Bonfoco, E *et al*. Glutamate-induced neuronal death: a succession of necrosis or apoptosis depending on mitochondrial function. *Neuron* **15**, 961-973, doi:0896-6273(95)90186-8 [pii] (1995).

38 Cheng, Y, Deshmukh, M, D'Costa, A *et al*. Caspase inhibitor affords neuroprotection with delayed administration in a rat model of neonatal hypoxic-ischemic brain injury. *J Clin Invest* **101**, 1992-1999, doi:10.1172/JCI2169 (1998).

39 Yu, SP, Yeh, CH, Gottron, F *et al*. Role of the outward delayed rectifier K+ current in ceramide-induced caspase activation and apoptosis in cultured cortical neurons. *J Neurochem* **73**, 933-941 (1999).

40 Yu, SP, Yeh, C, Strasser, U *et al*. NMDA receptor-mediated K+ efflux and neuronal apoptosis. *Science* **284**, 336-339 (1999).

41 Yu, SP, Yeh, CH, Sensi, SL *et al*. Mediation of neuronal apoptosis by enhancement of outward potassium current. *Science* **278**, 114-117 (1997).

42 Erecinska, M & Silver, IA. Ions and energy in mammalian brain. *Progress in Neurobiology* **43**, 37-71 (1994).

43 Waxman, SG, Black, JA, Ransom, BR *et al*. Anoxic injury of rat optic nerve: ultrastructural evidence for coupling between Na+ influx and Ca(2+)-mediated injury in myelinated CNS axons. *Brain Res* **644**, 197-204 (1994).

44 Friedman, JE & Haddad, GG. Anoxia induces an increase in intracellular sodium in rat central neurons in vitro. *Brain Res* **663**, 329-334 (1994).

45 Banasiak, KJ, Burenkova, O & Haddad, GG. Activation of voltage-sensitive sodium channels during oxygen deprivation leads to apoptotic neuronal death. *Neuroscience* **126**, 31-44, doi:10.1016/S0306-4522(03)00425-1 (2004).

46 Friedman, JE & Haddad, GG. Removal of extracellular sodium prevents anoxia-induced injury in freshly dissociated rat CA1 hippocampal neurons. *Brain Res* **641**, 57-64 (1994).

47 Yamasaki, Y, Kogure, K, Hara, H *et al*. The possible involvement of tetrodotoxin-sensitive ion channels in ischemic neuronal damage in the rat hippocampus. *Neurosci Lett* **121**, 251-254 (1991).

48 Lysko, PG, Webb, CL, Yue, TL *et al*. Neuroprotective effects of tetrodotoxin as a Na+ channel modulator and glutamate release inhibitor in cultured rat cerebellar neurons and in gerbil global brain ischemia. *Stroke* **25**, 2476-2482 (1994).

49 Goldberg, MP & Choi, DW. Intracellular free calcium increases in cultured cortical neurons deprived of oxygen and glucose. *Stroke* **21**, III75-77 (1990).

50 Wei, L, Yu, SP, Gottron, F *et al*. Potassium channel blockers attenuate hypoxia- and ischemia-induced neuronal death in vitro and in vivo. *Stroke* **34**, 1281-1286, doi:10.1161/01.STR.0000065828.18661.FE 01.STR.0000065828.18661.FE [pii] (2003).

51 Rothman, SM & Olney, JW. Excitotoxicity and the NMDA receptor--still lethal after eight years. *Trends Neurosci* **18**, 57-58 (1995).

52 Drejer, J, Benveniste, H, Diemer, N *et al*. Cellular origin of ischemia-induced glutamate release from brain tissue in vivo. *J Neurochem* **45**, 145-151 (1985).

53 Mitani, A, Andou, Y, Matsuda, S *et al*. Origin of ischemia-induced glutamate efflux in the CA1 field of the gerbil hippocampus: an in vivo brain microdialysis study. *J Neurochem* **63**, 2152-2164 (1994).

54 Simon, RP, Griffiths, T, Evans, MC *et al*. Calcium overload in selectively vulnerable neurons of the hippocampus during and after ischemia: an electron microscopy study in the rat. *J Cereb Blood Flow Metab* **4**, 350-361 (1984).

55 Rothman, S. Synaptic release of excitatory amino acid neurotransmitter mediates anoxic neuronal death. *J. Neurosci.* **4**, 1884-1891 (1984).

56 Stanika, RI, Winters, CA, Pivovarova, NB *et al*. Differential NMDA receptor-dependent calcium loading and mitochondrial dysfunction in CA1 vs. CA3 hippocampal neurons. *Neurobiol Dis* **37**, 403-411, doi:10.1016/j.nbd.2009.10.020 (2010).

57 Choi, DW. Glutamate neurotoxicity and diseases of the nervous system. *Neuron* **1**, 623-634, doi:0896-6273(88)90162-6 [pii] (1988).

58 Bouchelouche, P, Belhage, B, Frandsen, A *et al*. Glutamate receptor activation in cultured cerebellar granule cells increases cytosolic free Ca2+ by mobilization of cellular Ca2+ and activation of Ca2+ influx. *Exp Brain Res* **76**, 281-291 (1989).

59 Choi, DW. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. *Trends Neurosci* **11**, 465-469, doi:0166-2236(88)90200-7 [pii] (1988).

60 Tamura, A, Graham, DI, McCulloch, J *et al*. Focal cerebral ischaemia in the rat: 2. Regional cerebral blood flow determined by [14C]iodoantipyrine autoradiography following middle cerebral artery occlusion. *J Cereb Blood Flow Metab* **1**, 61-69, doi:10.1038/jcbfm.1981.7 (1981).

61 Tamura, A, Graham, DI, McCulloch, J *et al*. Focal cerebral ischaemia in the rat: 1. Description of technique and early neuropathological consequences following middle cerebral artery occlusion. *J Cereb Blood Flow Metab* **1**, 53-60, doi:10.1038/jcbfm.1981.6 (1981).

62 Longa, EZ, Weinstein, PR, Carlson, S *et al*. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke* **20**, 84-91 (1989).

63 O'Brien, MD & Waltz, AG. Transorbital approach for occluding the middle cerebral artery without craniectomy. *Stroke* **4**, 201-206 (1973).

64 Wei, L, Rovainen, CM & Woolsey, TA. Ministrokes in rat barrel cortex. *Stroke* **26**, 1459-1462 (1995).

65 Zhang, L, Schallert, T, Zhang, ZG *et al*. A test for detecting long-term sensorimotor dysfunction in the mouse after focal cerebral ischemia. *J Neurosci Methods* **117**, 207-214, doi:S0165027002001140 [pii] (2002).

66 Li, WL, Yu, SP, Ogle, ME *et al*. Enhanced neurogenesis and cell migration following focal ischemia and peripheral stimulation in mice. *Dev Neurobiol* **68**, 1474-1486, doi:10.1002/dneu.20674 (2008).

67 Whitaker, VR, Cui, L, Miller, S *et al*. Whisker stimulation enhances angiogenesis in the barrel cortex following focal ischemia in mice. *J Cerebr Blood F Met* **27**, 57-68, doi:Doi 10.1038/Sj.Jcbfm.9600318 (2007).

68 Eichenbaum, JW, Pevsner, PH, Pivawer, G *et al*. A murine photochemical stroke model with histologic correlates of apoptotic and nonapoptotic mechanisms. *J Pharmacol Toxicol Methods* **47**, 67-71, doi:S1056-8719(02)00215-0 [pii] (2002).

69 De Ryck, M, Keersmaekers, R, Duytschaever, H *et al*. Lubeluzole protects sensorimotor function and reduces infarct size in a photochemical stroke model in rats. *J Pharmacol Exp Ther* **279**, 748-758 (1996).

70 Chen, F, Suzuki, Y, Nagai, N *et al*. Rat cerebral ischemia induced with photochemical occlusion of proximal middle cerebral artery: a stroke model for MR imaging research. *MAGMA* **17**, 103-108, doi:10.1007/s10334-004-0061-9 (2004).

71 Zhang, RL, Chopp, M, Zhang, ZG *et al*. A rat model of focal embolic cerebral ischemia. *Brain Res* **766**, 83-92, doi:S0006-8993(97)00580-5 [pii] (1997).

72 Wang, CX, Yang, T & Shuaib, A. An improved version of embolic model of brain ischemic injury in the rat. *J Neurosci Methods* **109**, 147-151, doi:S0165027001004083 [pii] (2001).

73 Windle, V, Szymanska, A, Granter-Button, S *et al*. An analysis of four different methods of producing focal cerebral ischemia with endothelin-1 in the rat. *Exp Neurol* **201**, 324-334, doi:S0014-4886(06)00268-8 [pii] 10.1016/j.expneurol.2006.04.012 (2006).

74 Biernaskie, J, Corbett, D, Peeling, J *et al*. A serial MR study of cerebral blood flow changes and lesion development following endothelin-1-induced ischemia in rats. *Magn Reson Med* **46**, 827-830, doi:10.1002/mrm.1263 [pii] (2001).

75 Pulsinelli, WA & Brierley, JB. A new model of bilateral hemispheric ischemia in the unanesthetized rat. *Stroke* **10**, 267-272 (1979).

76 Smith, ML, Auer, RN & Siesjo, BK. The density and distribution of ischemic brain injury in the rat following 2-10 min of forebrain ischemia. *Acta Neuropathol* **64**, 319-332 (1984).

77 Greenberg, DA. Angiogenesis and stroke. *Drug News Perspect* **11**, 265-270 (1998).

78 Arvidsson, A, Collin, T, Kirik, D *et al*. Neuronal replacement from endogenous precursors in the adult brain after stroke. *Nat Med* **8**, 963-970, doi:10.1038/nm747 nm747 [pii] (2002).

79 Ohab, JJ & Carmichael, ST. Poststroke neurogenesis: emerging principles of migration and localization of immature neurons. *Neuroscientist* **14**, 369-380, doi:1073858407309545 [pii]
10.1177/1073858407309545 (2008).

80 Ohab, JJ, Fleming, S, Blesch, A *et al*. A neurovascular niche for neurogenesis after stroke. *J Neurosci* **26**, 13007-13016, doi:26/50/13007 [pii]
10.1523/JNEUROSCI.4323-06.2006 (2006).

81 Kornack, DR & Rakic, P. The generation, migration, and differentiation of olfactory neurons in the adult primate brain. *Proc Natl Acad Sci U S A* **98**, 4752-4757, doi:10.1073/pnas.081074998
98/8/4752 [pii] (2001).

82 Imitola, J, Raddassi, K, Park, KI *et al*. Directed migration of neural stem cells to sites of CNS injury by the stromal cell-derived factor 1alpha/CXC chemokine receptor 4 pathway. *Proc Natl Acad Sci U S A* **101**, 18117-18122, doi:0408258102 [pii]
10.1073/pnas.0408258102 (2004).

83 Kokaia, Z, Thored, P, Arvidsson, A *et al*. Regulation of stroke-induced neurogenesis in adult brain - Recent scientific progress. *Cerebral Cortex* **16**, I162-I167, doi:Doi 10.1093/Cercor/Bbhj174 (2006).

84 Xiong, Y, Mahmood, A & Chopp, M. Angiogenesis, neurogenesis and brain recovery of function following injury. *Curr Opin Investig Drugs* **11**, 298-308 (2010).

85 Smart, I. Subependymal Layer of Mouse Brain and Its Cell Production as Shown by Radioautography after Thymidine-H3 Injection. *Journal of Comparative Neurology* **116**, 325-& (1961).

86 Altman, J. Are new neurons formed in the brains of adult mammals? *Science* **135**, 1127-1128 (1962).

87 Eriksson, PS, Perfilieva, E, Bjork-Eriksson, T *et al*. Neurogenesis in the adult human hippocampus. *Nat Med* **4**, 1313-1317, doi:10.1038/3305 (1998).

88 Gould, E, Reeves, AJ, Fallah, M *et al*. Hippocampal neurogenesis in adult Old World primates. *Proc Natl Acad Sci U S A* **96**, 5263-5267 (1999).

89 Altman, J. Autoradiographic and histological studies of postnatal neurogenesis. IV. Cell proliferation and migration in the anterior forebrain, with special reference to persisting neurogenesis in the olfactory bulb. *J Comp Neurol* **137**, 433-457, doi:10.1002/cne.901370404 (1969).

90 Petreanu, L & Alvarez-Buylla, A. Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. *J Neurosci* **22**, 6106-6113, doi:20026588
22/14/6106 [pii] (2002).

91 Parent, JM. Injury-induced neurogenesis in the adult mammalian brain. *Neuroscientist* **9**, 261-272 (2003).

92 van Praag, H, Christie, BR, Sejnowski, TJ *et al*. Running enhances neurogenesis, learning, and long-term potentiation in mice. *Proc Natl Acad Sci U S A* **96**, 13427-13431 (1999).

93 van Praag, H, Kempermann, G & Gage, FH. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. *Nat Neurosci* **2**, 266-270, doi:10.1038/6368 (1999).

94 Chen, J, Zhang, C, Jiang, H *et al*. Atorvastatin induction of VEGF and BDNF promotes brain plasticity after stroke in mice. *J Cereb Blood Flow Metab* **25**, 281-290, doi:9600034 [pii]
10.1038/sj.jcbfm.9600034 (2005).

95 Belmadani, A, Tran, PB, Ren, D *et al*. Chemokines regulate the migration of neural progenitors to sites of neuroinflammation. *J Neurosci* **26**, 3182-3191, doi:26/12/3182 [pii]
10.1523/JNEUROSCI.0156-06.2006 (2006).

96 Jin, R, Yang, G & Li, G. Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. *J Leukoc Biol* **87**, 779-789, doi:jlb.1109766 [pii]
10.1189/jlb.1109766 (2010).

97 Lee, SR, Kim, HY, Rogowska, J *et al*. Involvement of matrix metalloproteinase in neuroblast cell migration from the subventricular zone after stroke. *J Neurosci* **26**, 3491-3495, doi:26/13/3491 [pii]
10.1523/JNEUROSCI.4085-05.2006 (2006).

98 Cybulska-Klosowicz, A, Liguz-Lecznar, M, Nowicka, D *et al*. Matrix metalloproteinase inhibition counteracts impairment of cortical experience-dependent plasticity after photothrombotic stroke. *Eur J Neurosci* **33**, 2238-2246, doi:10.1111/j.1460-9568.2011.07713.x (2011).

99 Kang, SS, Kook, JH, Hwang, S *et al*. Inhibition of matrix metalloproteinase-9 attenuated neural progenitor cell migration after photothrombotic ischemia. *Brain Res* **1228**, 20-26, doi:S0006-8993(08)01460-1 [pii]
10.1016/j.brainres.2008.06.056 (2008).

100 Widera, D, Holtkamp, W, Entschladen, F *et al*. MCP-1 induces migration of adult neural stem cells. *Eur J Cell Biol* **83**, 381-387 (2004).

101 Yan, YP, Sailor, KA, Lang, BT *et al*. Monocyte chemoattractant protein-1 plays a critical role in neuroblast migration after focal cerebral ischemia. *J Cereb Blood Flow Metab* **27**, 1213-1224, doi:9600432 [pii]
10.1038/sj.jcbfm.9600432 (2007).

102 Ekdahl, CT, Kokaia, Z & Lindvall, O. Brain inflammation and adult neurogenesis: the dual role of microglia. *Neuroscience* **158**, 1021-1029, doi:S0306-4522(08)00971-8 [pii]
10.1016/j.neuroscience.2008.06.052 (2009).

103 Covey, MV, Loporchio, D, Buono, KD *et al*. Opposite effect of inflammation on subventricular zone versus hippocampal precursors in brain injury. *Ann Neurol*, doi:10.1002/ana.22473 (2011).

104 Aarum, J, Sandberg, K, Haeberlein, SL *et al*. Migration and differentiation of neural precursor cells can be directed by microglia. *Proc Natl Acad Sci U S A* **100**, 15983-15988, doi:10.1073/pnas.2237050100
2237050100 [pii] (2003).

105 Gebicke-Haerter, PJ, Spleiss, O, Ren, LQ *et al*. Microglial chemokines and chemokine receptors. *Prog Brain Res* **132**, 525-532, doi:S0079-6123(01)32100-3 [pii]
10.1016/S0079-6123(01)32100-3 (2001).

106 Streit, WJ. Microglia as neuroprotective, immunocompetent cells of the CNS. *Glia* **40**, 133-139, doi:10.1002/glia.10154 (2002).

107 Li, M & Ransohoff, RM. Multiple roles of chemokine CXCL12 in the central nervous system: a migration from immunology to neurobiology. *Prog Neurobiol* **84**, 116-131, doi:S0301-0082(07)00212-2 [pii] 10.1016/j.pneurobio.2007.11.003 (2008).

108 Matthys, P, Hatse, S, Vermeire, K *et al*. AMD3100, a potent and specific antagonist of the stromal cell-derived factor-1 chemokine receptor CXCR4, inhibits autoimmune joint inflammation in IFN-gamma receptor-deficient mice. *J Immunol* **167**, 4686-4692 (2001).

109 Bleul, CC, Fuhlbrigge, RC, Casasnovas, JM *et al*. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med* **184**, 1101-1109 (1996).

110 Ma, Q, Jones, D, Borghesani, PR *et al*. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci U S A* **95**, 9448-9453 (1998).

111 Cruz-Orengo, L, Holman, DW, Dorsey, D *et al*. CXCR7 influences leukocyte entry into the CNS parenchyma by controlling abluminal CXCL12 abundance during autoimmunity. *Journal of Experimental Medicine* **208**, 327-339, doi:Doi 10.1084/Jem.20102010 (2011).

112 Huising, MO, Stet, RJ, Kruiswijk, CP *et al*. Molecular evolution of CXC chemokines: extant CXC chemokines originate from the CNS. *Trends Immunol* **24**, 307-313, doi:S1471490603001200 [pii] (2003).

113 Zou, YR, Kottmann, AH, Kuroda, M *et al*. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* **393**, 595-599, doi:10.1038/31269 (1998).

114 Rosu-Myles, M, Gallacher, L, Murdoch, B *et al*. The human hematopoietic stem cell compartment is heterogeneous for CXCR4 expression. *Proc Natl Acad Sci U S A* **97**, 14626-14631, doi:10.1073/pnas.97.26.14626 97/26/14626 [pii] (2000).

115 Pituch-Noworolska, A, Majka, M, Janowska-Wieczorek, A *et al*. Circulating CXCR4-positive stem/progenitor cells compete for SDF-1-positive niches in bone marrow, muscle and neural tissues: an alternative hypothesis to stem cell plasticity. *Folia Histochem Cytobiol* **41**, 13-21 (2003).

116 Ji, JF, He, BP, Dheen, ST *et al*. Expression of chemokine receptors CXCR4, CCR2, CCR5 and CX3CR1 in neural progenitor cells isolated from the subventricular zone of the adult rat brain. *Neurosci Lett* **355**, 236-240, doi:S0304394003013181 [pii] (2004).

117 Hatch, HM, Zheng, D, Jorgensen, ML *et al*. SDF-1alpha/CXCR4: a mechanism for hepatic oval cell activation and bone marrow stem cell recruitment to the injured liver of rats. *Cloning Stem Cells* **4**, 339-351, doi:10.1089/153623002321025014 (2002).

118 Crane, IJ, Wallace, CA, McKillop-Smith, S *et al*. CXCR4 receptor expression on human retinal pigment epithelial cells from the blood-retina barrier leads to chemokine secretion and migration in response to stromal cell-derived factor 1 alpha. *J Immunol* **165**, 4372-4378 (2000).

119 Guo, Y, Hangoc, G, Bian, H *et al*. SDF-1/CXCL12 enhances survival and chemotaxis of murine embryonic stem cells and production of primitive and

definitive hematopoietic progenitor cells. *Stem Cells* **23**, 1324-1332, doi:23/9/1324 [pii]
10.1634/stemcells.2005-0085 (2005).

120 Zhu, Y, Yu, T, Zhang, XC *et al*. Role of the chemokine SDF-1 as the meningeal attractant for embryonic cerebellar neurons. *Nat Neurosci* **5**, 719-720, doi:10.1038/nn881
nn881 [pii] (2002).

121 Liu, XS, Chopp, M, Santra, M *et al*. Functional response to SDF1 alpha through over-expression of CXCR4 on adult subventricular zone progenitor cells. *Brain Res* **1226**, 18-26, doi:S0006-8993(08)01374-7 [pii]
10.1016/j.brainres.2008.06.013 (2008).

122 Menezes, JR, Smith, CM, Nelson, KC *et al*. The division of neuronal progenitor cells during migration in the neonatal mammalian forebrain. *Mol Cell Neurosci* **6**, 496-508, doi:10.1006/mcne.1995.0002 (1995).

123 Smith, CM & Luskin, MB. Cell cycle length of olfactory bulb neuronal progenitors in the rostral migratory stream. *Dev Dyn* **213**, 220-227, doi:10.1002/(SICI)1097-0177(199810)213:2<220::AID-AJA7>3.0.CO;2-I [pii]
10.1002/(SICI)1097-0177(199810)213:2<220::AID-AJA7>3.0.CO;2-I (1998).

124 Zhang, R, Zhang, Z, Zhang, C *et al*. Stroke transiently increases subventricular zone cell division from asymmetric to symmetric and increases neuronal differentiation in the adult rat. *J Neurosci* **24**, 5810-5815, doi:10.1523/JNEUROSCI.1109-04.2004
24/25/5810 [pii] (2004).

125 Zhang, RL, Zhang, ZG, Zhang, L *et al*. Proliferation and differentiation of progenitor cells in the cortex and the subventricular zone in the adult rat after focal cerebral ischemia. *Neuroscience* **105**, 33-41, doi:S0306-4522(01)00117-8 [pii] (2001).

126 Kuge, A, Takemura, S, Kokubo, Y *et al*. Temporal profile of neurogenesis in the subventricular zone, dentate gyrus and cerebral cortex following transient focal cerebral ischemia. *Neurol Res* **31**, 969-976, doi:ner1753 [pii]
10.1179/174313209X383312 (2009).

127 Jin, K, Sun, Y, Xie, L *et al*. Directed migration of neuronal precursors into the ischemic cerebral cortex and striatum. *Mol Cell Neurosci* **24**, 171-189, doi:S1044743103001593 [pii] (2003).

128 Tonchev, AB, Yamashima, T, Sawamoto, K *et al*. Enhanced proliferation of progenitor cells in the subventricular zone and limited neuronal production in the striatum and neocortex of adult macaque monkeys after global cerebral ischemia. *J Neurosci Res* **81**, 776-788, doi:10.1002/jnr.20604 (2005).

129 Thored, P, Arvidsson, A, Cacci, E *et al*. Persistent production of neurons from adult brain stem cells during recovery after stroke. *Stem Cells* **24**, 739-747, doi:2005-0281 [pii]
10.1634/stemcells.2005-0281 (2006).

130 Parent, JM, Vexler, ZS, Gong, C *et al*. Rat forebrain neurogenesis and striatal neuron replacement after focal stroke. *Ann Neurol* **52**, 802-813, doi:10.1002/ana.10393 (2002).

131 Li, WL, Yu, SP, Ogle, ME *et al*. Enhanced Neurogenesis and Cell Migration Following Focal Ischemia and Peripheral Stimulation in Mice. *Developmental Neurobiology* **68**, 1474-1486, doi:Doi 10.1002/Dneu.20674 (2008).

132 Robin, AM, Zhang, ZG, Wang, L *et al*. Stromal cell-derived factor 1alpha mediates neural progenitor cell motility after focal cerebral ischemia. *J Cereb Blood Flow Metab* **26**, 125-134, doi:9600172 [pii] 10.1038/sj.jcbfm.9600172 (2006).

133 Hasselblatt, M, Jeibmann, A, Riesmeier, B *et al*. Granulocyte-colony stimulating factor (G-CSF) and G-CSF receptor expression in human ischemic stroke. *Acta Neuropathol* **113**, 45-51, doi:10.1007/s00401-006-0152-y (2007).

134 Stumm, RK, Rummel, J, Junker, V *et al*. A dual role for the SDF-1/CXCR4 chemokine receptor system in adult brain: Isoform-selective regulation of SDF-1 expression modulates CXCR4-dependent neuronal plasticity and cerebral leukocyte recruitment after focal ischemia. *Journal of Neuroscience* **22**, 5865-5878 (2002).

135 Miller, JT, Bartley, JH, Wimborne, HJ *et al*. The neuroblast and angioblast chemotaxic factor SDF-1 (CXCL12) expression is briefly up regulated by reactive astrocytes in brain following neonatal hypoxic-ischemic injury. *BMC Neurosci* **6**, 63, doi:10.1186/1471-2202-6-63 (2005).

136 Schonemeier, B, Schulz, S, Hoellt, V *et al*. Enhanced expression of the CXCl12/SDF-1 chemokine receptor CXCR7 after cerebral ischemia in the rat brain. *J Neuroimmunol* **198**, 39-45, doi:S0165-5728(08)00110-0 [pii] 10.1016/j.jneuroim.2008.04.010 (2008).

137 Shyu, WC, Lin, SZ, Yen, PS *et al*. Stromal cell-derived factor-1 alpha promotes neuroprotection, angiogenesis, and mobilization/homing of bone marrow-derived cells in stroke rats. *J Pharmacol Exp Ther* **324**, 834-849, doi:jpet.107.127746 [pii] 10.1124/jpet.107.127746 (2008).

138 Zhou, Y, Larsen, PH, Hao, C *et al*. CXCR4 is a major chemokine receptor on glioma cells and mediates their survival. *J Biol Chem* **277**, 49481-49487, doi:10.1074/jbc.M206222200 M206222200 [pii] (2002).

139 Rubin, JB, Kung, AL, Klein, RS *et al*. A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors. *Proc Natl Acad Sci U S A* **100**, 13513-13518, doi:10.1073/pnas.2235846100 2235846100 [pii] (2003).

140 Teicher, BA & Fricker, SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. *Clin Cancer Res* **16**, 2927-2931, doi:1078-0432.CCR-09-2329 [pii] 10.1158/1078-0432.CCR-09-2329 (2010).

141 Vlahakis, SR, Villasis-Keever, A, Gomez, T *et al*. G protein-coupled chemokine receptors induce both survival and apoptotic signaling pathways. *J Immunol* **169**, 5546-5554 (2002).

142 Ceradini, DJ, Kulkarni, AR, Callaghan, MJ *et al*. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nature Medicine* **10**, 858-864, doi:10.1038/nm1075 (2004).

143 Chau, M, McCullough, I, Yu, SP *et al*. in *Neuronal Migration: Disorders, Genetic Factors, and Treatment Options Neuroscience Research Progress* eds Andrew Girard & Luca Moreau) Ch. 5, (Nova Science Publishers, Inc., 2012).

144 Petit, I, Szyper-Kravitz, M, Nagler, A *et al*. G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol* **3**, 687-694, doi:10.1038/ni813
ni813 [pii] (2002).

145 Becker, AJ, Mc, CE & Till, JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. *Nature* **197**, 452-454 (1963).

146 Thomson, JA, Itskovitz-Eldor, J, Shapiro, SS *et al*. Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145-1147 (1998).

147 Theus, MH, Wei, L, Cui, L *et al*. In vitro hypoxic preconditioning of embryonic stem cells as a strategy of promoting cell survival and functional benefits after transplantation into the ischemic rat brain. *Exp Neurol* **210**, 656-670, doi:S0014-4886(07)00494-3 [pii]
.1016/j.expneurol.2007.12.020 (2008).

148 Takahashi, K, Okita, K, Nakagawa, M *et al*. Induction of pluripotent stem cells from fibroblast cultures. *Nat Protoc* **2**, 3081-3089, doi:nprot.2007.418 [pii]
10.1038/nprot.2007.418 (2007).

149 Takahashi, K, Tanabe, K, Ohnuki, M *et al*. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* **131**, 861-872, doi:S0092-8674(07)01471-7 [pii]
10.1016/j.cell.2007.11.019 (2007).

150 Chen, SJ, Chang, CM, Tsai, SK *et al*. Functional Improvement of Focal Cerebral Ischemia Injury by Subdural Transplantation of Induced Pluripotent Stem Cells with Fibrin Glue. *Stem Cells Dev*, doi:10.1089/scd.2009.0452 (2010).

151 van Velthoven, CT, Sheldon, RA, Kavelaars, A *et al*. Mesenchymal stem cell transplantation attenuates brain injury after neonatal stroke. *Stroke* **44**, 1426-1432, doi:10.1161/STROKEAHA.111.000326 (2013).

152 Wei, N, Yu, SP, Gu, X *et al*. Delayed Intranasal Delivery of Hypoxic-preconditioned Bone Marrow Mesenchymal Stem Cells Enhanced Cell Homing and Therapeutic Benefits after Ischemic Stroke in Mice. *Cell Transplant*, doi:10.3727/096368912X657251 (2012).

153 Bang, OY, Lee, JS, Lee, PH *et al*. Autologous mesenchymal stem cell transplantation in stroke patients. *Ann Neurol* **57**, 874-882, doi:10.1002/ana.20501 (2005).

154 Lee, JS, Hong, JM, Moon, GJ *et al*. A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells* **28**, 1099-1106, doi:10.1002/stem.430 (2010).

155 Suarez-Monteagudo, C, Hernandez-Ramirez, P, Alvarez-Gonzalez, L *et al*. Autologous bone marrow stem cell neurotransplantation in stroke patients. An open study. *Restorative neurology and neuroscience* **27**, 151-161, doi:10.3233/RNN-2009-0483 (2009).

156 Sanchez-Ramos, J, Song, S, Cardozo-Pelaez, F *et al*. Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp Neurol* **164**, 247-256, doi:10.1006/exnr.2000.7389 (2000).

157 Gutierrez-Fernandez, M, Rodriguez-Frutos, B, Ramos-Cejudo, J *et al*. Effects of intravenous administration of allogenic bone marrow- and adipose tissue-derived mesenchymal stem cells on functional recovery and brain repair markers in experimental ischemic stroke. *Stem Cell Res Ther* **4**, 11, doi:10.1186/scrt159 (2013).

158 Wei, L, Cui, L, Snider, BJ *et al*. Transplantation of embryonic stem cells overexpressing Bcl-2 promotes functional recovery after transient cerebral ischemia. *Neurobiol Dis* **19**, 183-193, doi:S0969-9961(04)00319-5 [pii]
10.1016/j.nbd.2004.12.016 (2005).

159 Mohamad, O, Drury-Stewart, D, Song, M *et al*. Vector-free and transgene-free human iPS cells differentiate into functional neurons and enhance functional recovery after ischemic stroke in mice. *PLoS One* **8**, e64160, doi:10.1371/journal.pone.0064160 (2013).

160 Buhnemann, C, Scholz, A, Bernreuther, C *et al*. Neuronal differentiation of transplanted embryonic stem cell-derived precursors in stroke lesions of adult rats. *Brain* **129**, 3238-3248, doi:10.1093/brain/awl261 (2006).

161 Hayashi, J, Takagi, Y, Fukuda, H *et al*. Primate embryonic stem cell-derived neuronal progenitors transplanted into ischemic brain. *J Cereb Blood Flow Metab* **26**, 906-914, doi:10.1038/sj.jcbfm.9600247 (2006).

162 Nonomura, T, Kubo, T, Oka, T *et al*. Signaling pathways and survival effects of BDNF and NT-3 on cultured cerebellar granule cells. *Dev Brain Res* **97**, 42-50, doi:Doi 10.1016/S0165-3806(96)00130-7 (1996).

163 Ye, L, Chang, JC, Lin, C *et al*. Induced pluripotent stem cells offer new approach to therapy in thalassemia and sickle cell anemia and option in prenatal diagnosis in genetic diseases. *Proc Natl Acad Sci U S A* **106**, 9826-9830, doi:0904689106 [pii]
10.1073/pnas.0904689106 (2009).

164 Kim, PG & Daley, GQ. Application of induced pluripotent stem cells to hematologic disease. *Cytotherapy* **11**, 980-989, doi:10.3109/14653240903348319 (2009).

165 Takahashi, K & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* **126**, 663-676, doi:S0092-8674(06)00976-7 [pii]
10.1016/j.cell.2006.07.024 (2006).

166 Wernig, M, Zhao, JP, Pruszak, J *et al*. Neurons derived from reprogrammed fibroblasts functionally integrate into the fetal brain and improve symptoms of rats with Parkinson's disease. *Proc Natl Acad Sci U S A* **105**, 5856-5861, doi:10.1073/pnas.0801677105 (2008).

167 Onorati, M, Camnasio, S, Binetti, M *et al*. Neuropotent self-renewing neural stem (NS) cells derived from mouse induced pluripotent stem (iPS) cells. *Mol Cell Neurosci* **43**, 287-295, doi:S1044-7431(09)00263-2 [pii]
10.1016/j.mcn.2009.12.002 (2010).

168 Reubinoff, BE, Itsykson, P, Turetsky, T *et al*. Neural progenitors from human embryonic stem cells. *Nat Biotechnol* **19**, 1134-1140, doi:10.1038/nbt1201-1134 nbt1201-1134 [pii] (2001).

169 Bain, G, Kitchens, D, Yao, M *et al*. Embryonic stem cells express neuronal properties in vitro. *Dev Biol* **168**, 342-357, doi:10.1006/dbio.1995.1085 S0012-1606(85)71085-8 [pii] (1995).

170 Jiang, M, Lv, L, Ji, H *et al*. Induction of pluripotent stem cells transplantation therapy for ischemic stroke. *Mol Cell Biochem* **354**, 67-75, doi:10.1007/s11010-011-0806-5 (2011).

171 Yu, J, Vodyanik, MA, Smuga-Otto, K *et al*. Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-1920, doi:1151526 [pii] 10.1126/science.1151526 (2007).

172 Oki, K, Tatarishvili, J, Wood, J *et al*. Human-induced pluripotent stem cells form functional neurons and improve recovery after grafting in stroke-damaged brain. *Stem Cells* **30**, 1120-1133, doi:10.1002/stem.1104 (2012).

173 Borlongan, CV, Tajima, Y, Trojanowski, JQ *et al*. Transplantation of cryopreserved human embryonal carcinoma-derived neurons (NT2N cells) promotes functional recovery in ischemic rats. *Exp Neurol* **149**, 310-321, doi:S0014-4886(97)96730-3 [pii] 10.1006/exnr.1997.6730 (1998).

174 Nishino, H & Borlongan, CV. Restoration of function by neural transplantation in the ischemic brain. *Prog Brain Res* **127**, 461-476 (2000).

175 Johnston, RE, Dillon-Carter, O, Freed, WJ *et al*. Trophic factor secreting kidney cell lines: in vitro characterization and functional effects following transplantation in ischemic rats. *Brain Res* **900**, 268-276, doi:S0006-8993(01)02327-7 [pii] (2001).

176 Chen, J, Sanberg, PR, Li, Y *et al*. Intravenous administration of human umbilical cord blood reduces behavioral deficits after stroke in rats. *Stroke* **32**, 2682-2688 (2001).

177 Li, Y, Chen, J, Wang, L *et al*. Treatment of stroke in rat with intracarotid administration of marrow stromal cells. *Neurology* **56**, 1666-1672 (2001).

178 Fischer, UM, Harting, MT, Jimenez, F *et al*. Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev* **18**, 683-692, doi:10.1089/scd.2008.0253 (2009).

179 Detante, O, Moisan, A, Dimastromatteo, J *et al*. Intravenous administration of 99mTc-HMPAO-labeled human mesenchymal stem cells after stroke: in vivo imaging and biodistribution. *Cell Transplant* **18**, 1369-1379, doi:10.3727/096368909X474230 (2009).

180 Corti, S, Locatelli, F, Strazzer, S *et al*. Modulated generation of neuronal cells from bone marrow by expansion and mobilization of circulating stem cells with in vivo cytokine treatment. *Exp Neurol* **177**, 443-452, doi:S0014488602980040 [pii] (2002).

181 Piao, CS, Gonzalez-Toledo, ME, Xue, YQ *et al*. The role of stem cell factor and granulocyte-colony stimulating factor in brain repair during chronic stroke. *J Cereb Blood Flow Metab* **29**, 759-770, doi:jcbfm2008168 [pii] 10.1038/jcbfm.2008.168 (2009).

182 Danielyan, L, Schafer, R, von Ameln-Mayerhofer, A *et al*. Therapeutic efficacy of intranasally delivered mesenchymal stem cells in a rat model of Parkinson disease. *Rejuvenation Res* **14**, 3-16, doi:10.1089/rej.2010.1130 (2011).

183 Danielyan, L, Schafer, R, von Ameln-Mayerhofer, A *et al*. Intranasal delivery of cells to the brain. *Eur J Cell Biol* **88**, 315-324, doi:10.1016/j.ejcb.2009.02.001 (2009).

184 van Velthoven, CT, Kavelaars, A, van Bel, F *et al*. Nasal administration of stem cells: a promising novel route to treat neonatal ischemic brain damage. *Pediatr Res* **68**, 419-422, doi:10.1203/PDR.0b013e3181f1c289 (2010).

185 Theus, MH, Wei, L, Cui, L *et al*. In vitro hypoxic preconditioning of embryonic stem cells as a strategy of promoting cell survival and functional benefits after transplantation into the ischemic rat brain. *Experimental Neurology* **210**, 656-670, doi:Doi 10.1016/J.Expneurol.2007.12.020 (2008).

186 Park, DS, Stefanis, L, Yan, CY *et al*. Ordering the cell death pathway. Differential effects of BCL2, an interleukin-1-converting enzyme family protease inhibitor, and other survival agents on JNK activation in serum/nerve growth factor-deprived PC12 cells. *J Biol Chem* **271**, 21898-21905 (1996).

187 Oh, JS, Liu, ML, Jin, HL *et al*. Effect of Bone Marrow Derived Mesenchymal Stem Cell Conditioned Media for Neurite Outgrowth in Human Ntera-2 Neurons. *Tissue Engineering and Regenerative Medicine* **6**, 562-567 (2009).

188 Nguyen, BK, Maltais, S, Perrault, LP *et al*. Improved function and myocardial repair of infarcted heart by intracoronary injection of mesenchymal stem cell-derived growth factors. *Journal of cardiovascular translational research* **3**, 547-558, doi:10.1007/s12265-010-9171-0 (2010).

189 Li, Y, Chen, J, Chen, XG *et al*. Human marrow stromal cell therapy for stroke in rat: neurotrophins and functional recovery. *Neurology* **59**, 514-523 (2002).

190 Bishop, ET, Bell, GT, Bloor, S *et al*. An in vitro model of angiogenesis: basic features. *Angiogenesis* **3**, 335-344 (1999).

191 Horie, N, Pereira, MP, Niizuma, K *et al*. Transplanted Stem Cell-Secreted VEGF Effects Post-Stroke Recovery, Inflammation, and Vascular Repair. *Stem Cells*, doi:10.1002/stem.584 (2011).

192 Gupta, SK, Lysko, PG, Pillarisetti, K *et al*. Chemokine receptors in human endothelial cells. Functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines. *J Biol Chem* **273**, 4282-4287 (1998).

193 Volin, MV, Joseph, L, Shockley, MS *et al*. Chemokine receptor CXCR4 expression in endothelium. *Biochem Biophys Res Commun* **242**, 46-53, doi:S0006-291X(97)97890-6 [pii]
10.1006/bbrc.1997.7890 (1998).

194 Yamaguchi, J, Kusano, KF, Masuo, O *et al*. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation* **107**, 1322-1328 (2003).

195 Salcedo, R, Wasserman, K, Young, HA *et al*. Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: In vivo neovascularization induced by stromal-derived factor-1alpha. *Am J Pathol* **154**, 1125-1135 (1999).

196 Li, Y, Lu, Z, Keogh, CL *et al*. Erythropoietin-induced neurovascular protection, angiogenesis, and cerebral blood flow restoration after focal ischemia in mice. *J Cereb Blood Flow Metab* **27**, 1043-1054, doi:10.1038/sj.jcbfm.9600417 (2007).

197 Wang, YM, Kilic, E, Kilic, U *et al*. VEGF overexpression induces post-ischaemic neuroprotection, but facilitates haemodynamic steal phenomena. *Brain* **128**, 52-63, doi:Doi 10.1093/Brain/Awh325 (2005).

198 Li, Y, Zhang, F, Nagai, NB *et al*. VEGF-B inhibits apoptosis via VEGFR-1-mediated suppression of the expression of BH3-only protein genes in mice and rats. *Journal of Clinical Investigation* **118**, 913-923, doi:Doi 10.1172/Jci33673 (2008).

199 Wang, L, Chopp, M, Gregg, SR *et al*. Neural progenitor cells treated with EPO induce angiogenesis through the production of VEGF. *J Cerebr Blood F Met* **28**, 1361-1368, doi:Doi 10.1038/Jcbfm.2008.32 (2008).

200 Zigova, T, Pencea, V, Wiegand, SJ *et al*. Intraventricular administration of BDNF increases the number of newly generated neurons in the adult olfactory bulb. *Molecular and cellular neurosciences* **11**, 234-245, doi:10.1006/mcne.1998.0684 (1998).

201 Pencea, V, Bingaman, KD, Wiegand, SJ *et al*. Infusion of brain-derived neurotrophic factor into the lateral ventricle of the adult rat leads to new neurons in the parenchyma of the striatum, septum, thalamus, and hypothalamus. *J Neurosci* **21**, 6706-6717 (2001).

202 Liu, H, Honmou, O, Harada, K *et al*. Neuroprotection by PlGF gene-modified human mesenchymal stem cells after cerebral ischaemia. *Brain* **129**, 2734-2745, doi:10.1093/brain/awl207 (2006).

203 Onda, T, Honmou, O, Harada, K *et al*. Therapeutic benefits by human mesenchymal stem cells (hMSCs) and Ang-1 gene-modified hMSCs after cerebral ischemia. *J Cereb Blood Flow Metab* **28**, 329-340, doi:10.1038/sj.jcbfm.9600527 (2008).

204 Kurozumi, K, Nakamura, K, Tamiya, T *et al*. Mesenchymal stem cells that produce neurotrophic factors reduce ischemic damage in the rat middle cerebral artery occlusion model. *Molecular therapy : the journal of the American Society of Gene Therapy* **11**, 96-104, doi:10.1016/j.ymthe.2004.09.020 (2005).

205 Park, KI. Transplantation of neural stem cells: cellular & gene therapy for hypoxic-ischemic brain injury. *Yonsei Med J* **41**, 825-835 (2000).

206 Dahl, NA & Balfour, WM. Prolonged Anoxic Survival Due to Anoxia Pre-Exposure: Brain Atp, Lactate, and Pyruvate. *Am J Physiol* **207**, 452-456 (1964).

207 Wei, L, Fraser, JL, Lu, ZY *et al*. Transplantation of hypoxia preconditioned bone marrow mesenchymal stem cells enhances angiogenesis and neurogenesis after cerebral ischemia in rats. *Neurobiology of Disease* **46**, 635-645, doi:Doi 10.1016/J.Nbd.2012.03.002 (2012).

208 Francis, KR & Wei, L. Human embryonic stem cell neural differentiation and enhanced cell survival promoted by hypoxic preconditioning. *Cell Death Dis* **1**, e22, doi:cddis200922 [pii]
10.1038/cddis.2009.22 (2010).

209 Bain, G, Ray, WJ, Yao, M *et al*. Retinoic acid promotes neural and represses mesodermal gene expression in mouse embryonic stem cells in culture. *Biochem Biophys Res Commun* **223**, 691-694, doi:10.1006/bbrc.1996.0957 (1996).

210 Bottenstein, JE & Sato, GH. Growth of a Rat Neuroblastoma Cell Line in Serum-Free Supplemented Medium. *P Natl Acad Sci USA* **76**, 514-517, doi:Doi 10.1073/Pnas.76.1.514 (1979).

211 Wei, L, Han, BH, Li, Y *et al*. Cell death mechanism and protective effect of erythropoietin after focal ischemia in the whisker-barrel cortex of neonatal rats. *J Pharmacol Exp Ther* **317**, 109-116, doi:10.1124/jpet.105.094391 (2006).

212 Schaar, KL, Brenneman, MM & Savitz, SI. Functional assessments in the rodent stroke model. *Experimental & translational stroke medicine* **2**, 13, doi:10.1186/2040-7378-2-13 (2010).

213 Hua, Y, Schallert, T, Keep, RF *et al*. Behavioral tests after intracerebral hemorrhage in the rat. *Stroke* **33**, 2478-2484 (2002).

214 Bouet, V, Boulouard, M, Toutain, J *et al*. The adhesive removal test: a sensitive method to assess sensorimotor deficits in mice. *Nat Protoc* **4**, 1560-1564, doi:nprot.2009.125 [pii]
10.1038/nprot.2009.125 (2009).

215 Agrawal, N, Johnston, SC, Wu, YW *et al*. Imaging Data Reveal a Higher Pediatric Stroke Incidence Than Prior US Estimates. *Stroke* **40**, 3415-3421, doi:Doi 10.1161/Strokeaha.109.564633 (2009).

216 Ganesan, V, Prengler, M, McShane, MA *et al*. Investigation of risk factors in children with arterial ischemic stroke. *Ann Neurol* **53**, 167-173, doi:10.1002/ana.10423 (2003).

217 Lynch, JK, Hirtz, DG, DeVeber, G *et al*. Report of the National Institute of Neurological Disorders and Stroke workshop on perinatal and childhood stroke. *Pediatrics* **109**, 116-123 (2002).

218 Monagle, P, Adams, M, Mahoney, M *et al*. Outcome of pediatric thromboembolic disease: a report from the Canadian Childhood Thrombophilia Registry. *Pediatr Res* **47**, 763-766 (2000).

219 Wusthoff, CJ, Kessler, SK, Vossough, A *et al*. Risk of later seizure after perinatal arterial ischemic stroke: a prospective cohort study. *Pediatrics* **127**, e1550-1557, doi:10.1542/peds.2010-1577 (2011).

220 Gardner, MA, Hills, NK, Sidney, S *et al*. The 5-year direct medical cost of neonatal and childhood stroke in a population-based cohort. *Neurology* **74**, 372-378, doi:10.1212/WNL.0b013e3181cbcd48 (2010).

221 Dimos, JT, Rodolfa, KT, Niakan, KK *et al*. Induced pluripotent stem cells generated from patients with ALS can be differentiated into motor neurons. *Science* **321**, 1218-1221, doi:1158799 [pii]
10.1126/science.1158799 (2008).

222 Chambers, SM, Fasano, CA, Papapetrou, EP *et al*. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. *Nat Biotechnol* **27**, 275-280, doi:nbt.1529 [pii]
10.1038/nbt.1529 (2009).

223 Karumbayaram, S, Novitch, BG, Patterson, M *et al*. Directed differentiation of human-induced pluripotent stem cells generates active motor neurons. *Stem Cells* **27**, 806-811, doi:10.1002/stem.31 (2009).

224 Drury-Stewart, D, Song, M, Mohamad, O *et al*. Highly efficient differentiation of neural precursors from human embryonic stem cells and benefits of transplantation after ischemic stroke in mice. *Stem Cell Res Ther* **4**, 93, doi:10.1186/scrt292 (2013).

225 Frank, J, Carroll, CM, Aaranson, K *et al*. Ischemia increases the angiogenic potency of basic fibroblast growth factor (FGF-2). *Microsurgery* **17**, 452-456; discussion 457-458, doi:10.1002/(SICI)1098-2752(1996)17:8<452::AID-MICR7>3.0.CO;2-G [pii] 10.1002/(SICI)1098-2752(1996)17:8<452::AID-MICR7>3.0.CO;2-G (1996).

226 Wang, L, Chopp, M, Gregg, SR *et al*. Neural progenitor cells treated with EPO induce angiogenesis through the production of VEGF. *J Cereb Blood Flow Metab* **28**, 1361-1368, doi:jcbfm200832 [pii] 10.1038/jcbfm.2008.32 (2008).

227 Kobayashi, T, Ahlenius, H, Thored, P *et al*. Intracerebral infusion of glial cell line-derived neurotrophic factor promotes striatal neurogenesis after stroke in adult rats. *Stroke* **37**, 2361-2367, doi:10.1161/01.STR.0000236025.44089.e1 (2006).

228 Kawai, H, Yamashita, T, Ohta, Y *et al*. Tridermal tumorigenesis of induced pluripotent stem cells transplanted in ischemic brain. *J Cereb Blood Flow Metab*, doi:jcbfm201032 [pii] 10.1038/jcbfm.2010.32 (2010).

229 Mellado, M, Rodriguez-Frade, JM, Manes, S *et al*. Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. *Annual review of immunology* **19**, 397-421, doi:10.1146/annurev.immunol.19.1.397 (2001).

230 Yoshida, M, Ishikawa, M, Izumi, H *et al*. Store-operated calcium channel regulates the chemotactic behavior of ascidian sperm. *Proc Natl Acad Sci U S A* **100**, 149-154, doi:10.1073/pnas.0135565100 (2003).

231 Wang, JF, Park, IW & Groopman, JE. Stromal cell-derived factor-1alpha stimulates tyrosine phosphorylation of multiple focal adhesion proteins and induces migration of hematopoietic progenitor cells: roles of phosphoinositide-3 kinase and protein kinase C. *Blood* **95**, 2505-2513 (2000).

232 Zhang, XF, Wang, JF, Matczak, E *et al*. Janus kinase 2 is involved in stromal cell-derived factor-1alpha-induced tyrosine phosphorylation of focal adhesion proteins and migration of hematopoietic progenitor cells. *Blood* **97**, 3342-3348 (2001).

233 Rabbany, SY, Pastore, J, Yamamoto, M *et al*. Continuous delivery of stromal cell-derived factor-1 from alginate scaffolds accelerates wound healing. *Cell Transplant* **19**, 399-408, doi:10.3727/096368909X481782 (2010).

234 Zhang, M, Mal, N, Kiedrowski, M *et al*. SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. *Faseb J* **21**, 3197-3207, doi:10.1096/fj.06-6558com (2007).

235 Hong, S, Hwang, DY, Yoon, S *et al*. Functional analysis of various promoters in lentiviral vectors at different stages of in vitro differentiation of mouse embryonic

stem cells. *Molecular therapy : the journal of the American Society of Gene Therapy* **15**, 1630-1639, doi:10.1038/sj.mt.6300251 (2007).

236 Wang, R, Liang, J, Jiang, H *et al*. Promoter-dependent EGFP expression during embryonic stem cell propagation and differentiation. *Stem Cells Dev* **17**, 279-289, doi:10.1089/scd.2007.0084 (2008).

237 Chu, G, Hayakawa, H & Berg, P. Electroporation for the efficient transfection of mammalian cells with DNA. *Nucleic acids research* **15**, 1311-1326 (1987).

238 Qin, JY, Zhang, L, Clift, KL *et al*. Systematic comparison of constitutive promoters and the doxycycline-inducible promoter. *PLoS One* **5**, e10611, doi:10.1371/journal.pone.0010611 (2010).

239 Arthur, DB, Akassoglou, K & Insel, PA. P2Y2 receptor activates nerve growth factor/TrkA signaling to enhance neuronal differentiation. *Proc Natl Acad Sci U S A* **102**, 19138-19143, doi:10.1073/pnas.0505913102 (2005).

240 Islam, O, Gong, X, Rose-John, S *et al*. Interleukin-6 and neural stem cells: more than gliogenesis. *Mol Biol Cell* **20**, 188-199, doi:10.1091/mbc.E08-05-0463 (2009).

241 Schwartz, CM, Tavakoli, T, Jamias, C *et al*. Stromal factors SDF1alpha, sFRP1, and VEGFD induce dopaminergic neuron differentiation of human pluripotent stem cells. *J Neurosci Res* **90**, 1367-1381, doi:10.1002/jnr.23064 (2012).

242 Kawasaki, H, Mizuseki, K, Nishikawa, S *et al*. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron* **28**, 31-40 (2000).

243 Morizane, A, Takahashi, J, Takagi, Y *et al*. Optimal conditions for in vivo induction of dopaminergic neurons from embryonic stem cells through stromal cell-derived inducing activity. *J Neurosci Res* **69**, 934-939, doi:10.1002/jnr.10363 (2002).

244 Vazin, T, Chen, J, Lee, CT *et al*. Assessment of stromal-derived inducing activity in the generation of dopaminergic neurons from human embryonic stem cells. *Stem Cells* **26**, 1517-1525, doi:10.1634/stemcells.2008-0039 (2008).

245 Shen, L, Gao, Y, Qian, J *et al*. A novel mechanism for endothelial progenitor cells homing: The SDF-1/CXCR4-Rac pathway may regulate endothelial progenitor cells homing through cellular polarization. *Medical hypotheses* **76**, 256-258, doi:10.1016/j.mehy.2010.10.014 (2011).

246 Thored, P, Wood, J, Arvidsson, A *et al*. Long-term neuroblast migration along blood vessels in an area with transient angiogenesis and increased vascularization after stroke. *Stroke* **38**, 3032-3039, doi:STROKEAHA.107.488445 [pii] 10.1161/STROKEAHA.107.488445 (2007).

247 Hill, WD, Hess, DC, Martin-Studdard, A *et al*. SDF-1 (CXCL12) is upregulated in the ischemic penumbra following stroke: association with bone marrow cell homing to injury. *J Neuropathol Exp Neurol* **63**, 84-96 (2004).

248 Janowski, M. Functional diversity of SDF-1 splicing variants. *Cell adhesion & migration* **3**, 243-249 (2009).

249 Guan, K, Chang, H, Rolletschek, A *et al*. Embryonic stem cell-derived neurogenesis. Retinoic acid induction and lineage selection of neuronal cells. *Cell Tissue Res* **305**, 171-176 (2001).

250 Englund, U, Bjorklund, A, Wictorin, K *et al*. Grafted neural stem cells develop into functional pyramidal neurons and integrate into host cortical circuitry. *Proc Natl Acad Sci U S A* **99**, 17089-17094, doi:10.1073/pnas.252589099 (2002).

251 Gomperts, BN, Belperio, JA, Rao, PN *et al*. Circulating progenitor epithelial cells traffic via CXCR4/CXCL12 in response to airway injury. *J Immunol* **176**, 1916-1927 (2006).

252 Bhattacharyya, BJ, Banisadr, G, Jung, H *et al*. The chemokine stromal cell-derived factor-1 regulates GABAergic inputs to neural progenitors in the postnatal dentate gyrus. *J Neurosci* **28**, 6720-6730, doi:10.1523/JNEUROSCI.1677-08.2008 (2008).

253 Callewaere, C, Banisadr, G, Desarmenien, MG *et al*. The chemokine SDF-1/CXCL12 modulates the firing pattern of vasopressin neurons and counteracts induced vasopressin release through CXCR4. *Proc Natl Acad Sci U S A* **103**, 8221-8226, doi:10.1073/pnas.0602620103 (2006).

254 Heinisch, S & Kirby, LG. SDF-1alpha/CXCL12 enhances GABA and glutamate synaptic activity at serotonin neurons in the rat dorsal raphe nucleus. *Neuropharmacology* **58**, 501-514, doi:10.1016/j.neuropharm.2009.08.022 (2010).

255 Burns, JM, Summers, BC, Wang, Y *et al*. A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *Journal of Experimental Medicine* **203**, 2201-2213, doi:Doi 10.1084/Jem.20052144 (2006).

256 Whitaker, VR, Cui, L, Miller, S *et al*. Whisker stimulation enhances angiogenesis in the barrel cortex following focal ischemia in mice. *J Cereb Blood Flow Metab* **27**, 57-68, doi:9600318 [pii]
10.1038/sj.jcbfm.9600318 (2007).

257 Zhong, J, Chan, A, Morad, L *et al*. Hydrogel matrix to support stem cell survival after brain transplantation in stroke. *Neurorehabilitation and neural repair* **24**, 636-644, doi:10.1177/1545968310361958 (2010).

258 Dvorak, HF, Detmar, M, Claffey, KP *et al*. Vascular permeability factor/vascular endothelial growth factor: an important mediator of angiogenesis in malignancy and inflammation. *International archives of allergy and immunology* **107**, 233-235 (1995).

259 Manoonkitiwongsa, PS, Schultz, RL, Whitter, EF *et al*. Contraindications of VEGF-based therapeutic angiogenesis: effects on macrophage density and histology of normal and ischemic brains. *Vascul Pharmacol* **44**, 316-325, doi:10.1016/j.vph.2006.01.008 (2006).